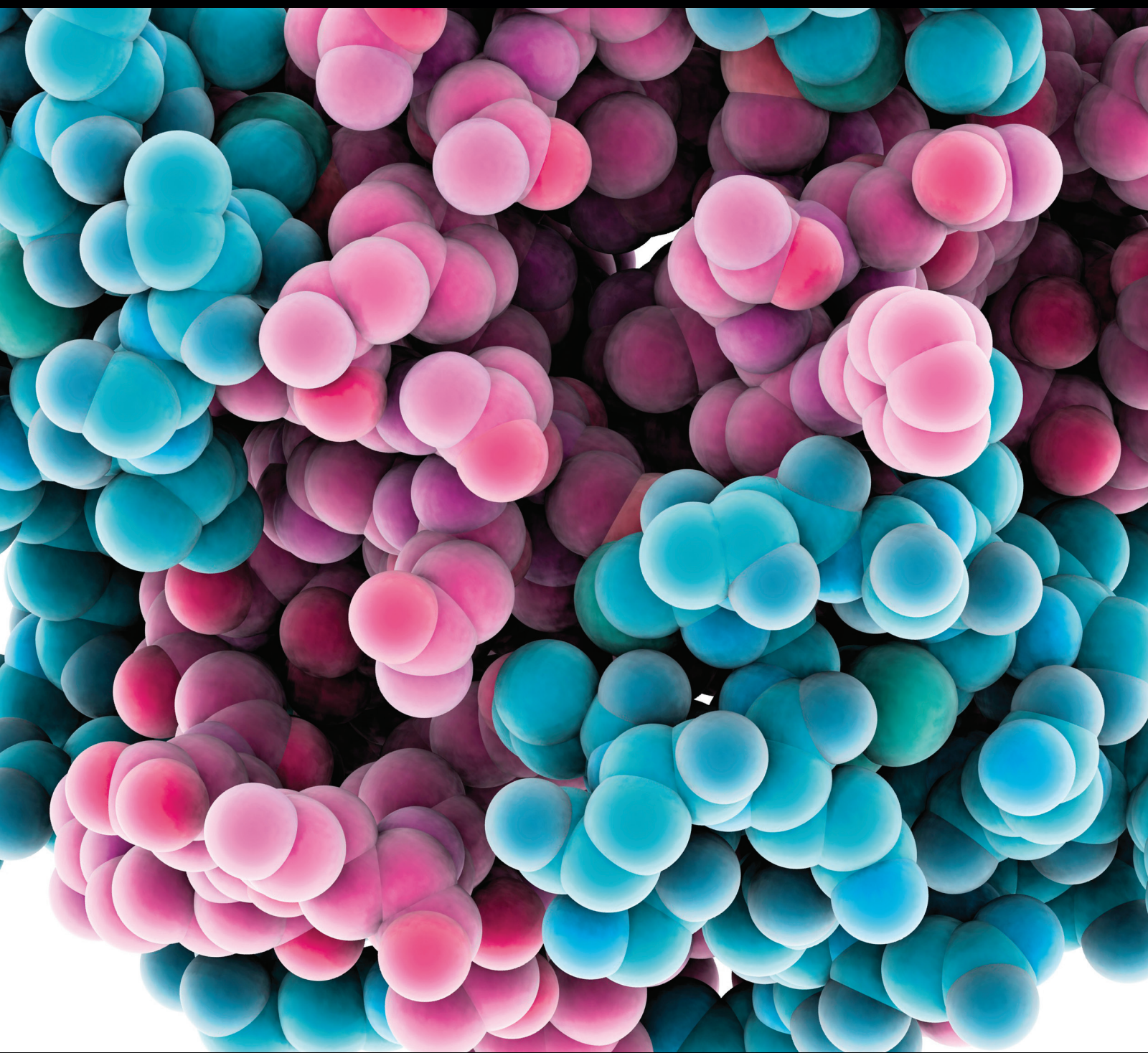
Journal of Diabetes Research

Complications of Diabetes 2016

Guest Editors: Konstantinos Papatheodorou, Nikolaos Papanas, Maciej Banach, Dimitrios Papazoglou, and Michael Edmonds



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Editorial Board

Contents

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Editorial

Complications of Diabetes 2016

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The prevalence of diabetes (DM) is constantly increasing worldwide at an alarming rate. According to the International Diabetes Federation in 2015, an estimated 415 million people globally were suffering from this condition [1]. Complications of DM account for increased morbidity, disability, and mortality and represent a threat for the economies of all countries, especially the developing ones [2]. The present special issue has been devoted to the recent progress in our understanding of diabetic complications, including the underlying molecular mechanisms, new diagnostic tools that facilitate early diagnosis, and novel treatment options. It consists of 20 articles covering 5 thematic areas: (a) epidemiology and pathogenesis of diabetic complications, (b) microvascular complications, (c) macrovascular complications, (d) miscellaneous complications, and (e) treatment options.

(a) Epidemiology and Pathogenesis of Diabetic Complications. There is growing evidence that the underlying mechanisms in the pathogenesis of diabetic complications include certain genetic and epigenetic modifications, nutritional factors, and sedentary lifestyle [3]. In a paper of this special issue entitled "Epigenetic Studies Point to DNA Replication/Repair Genes as a Basis for the Heritable Nature of Long Term Complications in Diabetes," A. A. Leontovich et al., using a zebrafish diabetic model, have explored the role of epigenetic mechanisms on the persistence of diabetic complications even after euglycemic control is achieved, a condition known as metabolic memory. They found that DNA-methylation, in or near genes belonging to the DNA replication/DNA metabolism process group, might play a key role in this process. Regarding basic risk factors for macro- and microvascular complications, the Irish Longitudinal Study on Ageing (TILDA), as M. L. Tracey et al. describe in their article "Risk Factors for Macro- and Microvascular Complications among Older Adults with Diagnosed Type 2 Diabetes: Findings from The Irish Longitudinal Study on Ageing," has recognized ageing, male gender, smoking, low level of physical activity, and high cholesterol as independent predictors of macrovascular complications. Conversely, smoking, hypertension, and duration of DM over 10 years proved to be predictive factors for microvascular complications.

(b) Microvascular Complications. Diabetic nephropathy, neuropathy, and retinopathy are the main microvascular complications induced by chronic hyperglycemia via several mechanisms such as the production of advanced glycation end products (AGEs), the creation of a proinflammatory microenvironment, and the induction of oxidative stress [4, 5].

Four articles in this special issue focus on diabetic nephropathy (DN). The first, by K. Sawada et al. entitled "Upregulation of $\alpha 3\beta 1$-Integrin in Podocytes in Early-Stage Diabetic Nephropathy" shed light on the mechanism of podocyte detachment from the glomerular basement membrane, which is considered to be a key factor in the development of DN. The authors conclude that the early stages of this procedure are mediated by an upregulation of $\alpha 3\beta 1$-integrin in podocytes. In the second article about DN entitled "Oxidative Stress in Diabetic Nephropathy with Early Chronic Kidney Disease," A. G. Miranda-Díaz et al. have reviewed the effects of hyperglycemia-induced production of reactive oxygen species (ROS) on the renin-angiotensin system and the signaling pathway of the transforming growth

factor-beta (TGF-β). They have concluded that oxidative stress leads to the production of chronic inflammation and the glomerular and tubular hypertrophy, which characterize the early stages of DN. Turning their attention to the diagnosis of early diabetic nephropathy, C. Gluhovschi et al., in another paper of this issue titled "Urinary Biomarkers in the Assessment of Early Diabetic Nephropathy," have attempted to review novel biomarkers indicating renal injury such as transferrin, ceruloplasmin, podocalyxin, and VEGF. These markers can detect renal injury even before the presence of microalbuminuria, which still remains the most valid biomarker for DN in clinical practice. The last paper on the same topic, by X. Li et al., entitled "Histone Acetylation and its Modifiers in the Pathogenesis of Diabetic Nephropathy," provides an overview of the potential involvement of epigenetic mechanisms, such as histone acetylation and other cellular processes in the development and progression of DN. It may be hoped that these mechanisms can help towards defining new therapeutic approaches for this microvascular complication of DM.

Three more articles have been included in the present special issue referring to diabetic retinopathy and neuropathy. The article entitled "Diabetic Retinopathy Is Strongly Predictive of Cardiovascular Autonomic Neuropathy in Type 2 Diabetes" has evaluated risk factors for cardiovascular autonomic neuropathy (CAN) in patients with T2DM. In this article, C.-C. Huang et al., using the deep breathing test, the Valsalva maneuver method, and the Composite Autonomic Scoring Scale to estimate the severity of autonomic neuropathy, found that diabetic retinopathy is the most significant predictive factor for CAN. In a further work, L. Forga et al. have conducted an observational, retrospective study in order to identify risk factors for the development of diabetic retinopathy (DR) in patients with type 1 DM. In their study entitled "Influence of Age at Diagnosis and Time-Dependent Risk Factors on the Development of Diabetic Retinopathy in Patients with Type 1 Diabetes," they maintain that age at onset of type 1 DM, indexes of glycemic control, HDL-cholesterol levels, and diastolic blood pressure are all parameters predicting DR. In this regard, the study "Heart Rate Variability as Early Biomarker for the Evaluation of Diabetes Mellitus Progress" by R. E. Arroyo-Carmona et al. has used heart rate variability (HRV) as a tool to identify early diabetic complications and progress of DM in streptozotocin-induced diabetic mice.

(c) Macrovascular Complications. Atherosclerosis is more common in people with DM than in those without. For example, DM increases the risk for stroke in people aged 20 to 65 years more than 5 times [6]. The present special issue includes articles on macrovascular complications of DM as well. J. Zhang et al. in the article entitled "Coronary Plaque Characteristics Assessed by 256-Slice Coronary CT Angiography and Association with High-Sensitivity C-Reactive Protein in Symptomatic Patients with Type 2 Diabetes" have performed a coronary Computed Tomography Angiography to evaluate coronary plaque subtypes and luminal narrowing in patients with and without type 2 DM. They report that patients with DM are more prone to have significant stenosis with calcified plaques and such findings are accompanied by higher hs-CRP levels. Moreover, in a review article entitled "The Role of AGE/RAGE Signaling in Diabetes-Mediated Vascular Calcification," A. M. Kay et al. emphasize the key role of AGE/RAGE signaling on the promotion of DM-mediated vascular calcification. In this process, many intracellular signaling pathways contribute to increased oxidative stress, which in turn leads to deposition of hydroxyapatite minerals into the extracellular matrix and vascular calcification. Furthermore, M. Samoš et al. in their work "The Impact of Type 2 Diabetes on the Efficacy of ADP Receptor Blockers in Patients with Acute ST Elevation Myocardial Infarction: A Pilot Prospective Study" have presented data from a prospective study that aimed to investigate platelet reactivity in patients with acute ST elevation myocardial infarction (STEMI) with or without T2DM, who have been treated with adenosine diphosphate (ADP) receptor blockers. Of note, this study has shown no difference between the two groups regarding platelet reactivity and the number of nonresponders to ADP receptor blockers. The last article of this thematic area is a retrospective quantitative study conducted in Australia. As B. T. Rodrigues et al. describe in their manuscript entitled "Prevalence and Risk Factors for Diabetic Lower Limb Amputation: A Clinic-Based Case Control Study," ethnicity has been recognized as an independent risk factor for lower limb amputation in patients with diabetic foot, among whom indigenous Australians were most commonly affected.

(d) Miscellaneous Complications. Diabetic cardiomyopathy is a specific complication that develops independently of coronary artery disease or hypertension and it is possible to lead to increased morbidity and mortality [7]. The aim of the study "Assessment of Left Ventricular Structural Remodelling in Patients with Diabetic Cardiomyopathy by Cardiovascular Magnetic Resonance" by Y. Shang et al. was to evaluate the structural remodeling of left ventricular (LV) mass in patients with diabetic cardiomyopathy (DCM) using cardiovascular magnetic resonance (CMR). The authors contend that CMR can be a valid tool to estimate LV remodeling and its severity in patients with DCM. Y. Yu et al. in their article entitled "The Protective Effect of Low Dose Ethanol on Myocardial Fibrosis through Downregulating the JNK Signaling Pathway in Diabetic Rats" have explored the protective role of low dose ethanol on myocardial fibrosis in diabetic rats. In this study, low dose ethanol consumption was associated with lower mean arterial pressure, lower heart rate, high hydroxyproline content, and collagen volume fraction in myocardial tissue, together with decreased expression of ALDH2 and downregulation of the JNK pathway. Finally, in the review paper "Molecular and Electrophysiological Mechanisms Underlying Cardiac Arrhythmogenesis in Diabetes Mellitus," G. Tse et al. discuss in detail the role of several cardiac factors (e.g., abnormalities in conduction or repolarization, electrophysiological, and structural remodeling) on arrhythmogenesis in patients with DM. They suggest that deeper investigation of these mechanisms can help towards defining new target molecules for potential future antiarrhythmic therapy for patients with DM.

(e) Treatment Options. The last thematic area covered by the present special issue relates to novel therapeutic options and it comprises four articles. In the first entitled "The Yin and Yang of the Opioid Growth Regulatory System: Focus on Diabetes: The Lorenz E. Zimmerman Tribute Lecture," J. W. Sassani et al. provide an extensive overview of the role of the Opioid Growth Regulatory System on the development of diabetic complications. The authors have summarized all recent evidence indicating that certain pharmaceutical modifications in the function of this system can have profitable effects on diabetic animals. Clearly, there is a lot to learn about these intricate issues in the future. The second manuscript, "Implementation of a Diabetes Educator-Care Model to Reduce Paediatric Admission for Diabetic Ketoacidosis" by A. Deeb et al., has evaluated a diabetes educator-care model aiming to reduce the frequency of hospital admission of children and adolescents due to Diabetic Ketoacidosis (DKA). The authors have demonstrated that this model was an effective and sustainable measure for DM treatment achieving a significant reduction in the admission rate for DKA. L. Voroneanu et al. in their article entitled "Silymarin in Type 2 Diabetes Mellitus: A Systematic Review and Meta-Analysis of Randomized Controlled Trials" have conducted a comprehensive review and meta-analysis to evaluate the efficacy and safety of silymarin administration in patients with T2DM. They have found that this extract of milk thistle is an efficient and safe antidiabetic agent that might also have beneficial effects on renal function. However, significant heterogeneity and low quality of the available evidence were noted and lead to the need for further investigation of this issue. The final study pertains to the treatment of diabetic foot ulcers. M. Janka-Zires et al. in their article titled "Topical Administration of Pirfenidone Increases Healing of Chronic Diabetic Foot Ulcers: A Randomized Crossover Study" have conducted a randomized crossover study to assess the effect of topical administration of pirfenidone on noninfected chronic diabetic foot ulcers. Their findings confirm that the healing of these ulcers improves significantly by topical addition of pirfenidone.

Acknowledgments

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Konstantinos Papatheodorou
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Review Article

The Yin and Yang of the Opioid Growth Regulatory System: Focus on Diabetes—The Lorenz E. Zimmerman Tribute Lecture

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The Opioid Growth Regulatory System consists of opioid growth factor (OGF), [Met^5]-enkephalin, and its unique receptor (OGFr). OGF inhibits cell division when bound to OGFr. Conversely, blockade of the interaction of OGF and OGFr, using the potent, long-acting opioid receptor antagonist, naltrexone (NTX), results in increased DNA synthesis and cell division. The authors have demonstrated both *in vitro* and *in vivo* that the addition of exogenous OGF or an increase in available OGFr decreases corneal epithelial cell division and wound healing. Conversely, blockade of the OGF-OGFr interaction by NTX or a decrease in the production of the OGFr increases corneal epithelial cell division and facilitates corneal epithelial wound healing. The authors also have demonstrated that depressed corneal and cutaneous wound healing, dry eye, and abnormal corneal sensitivity in type 1 and type 2 diabetes in animals can be reversed by OGF-OGFr blockade by NTX. Thus, the function of the Opioid Growth Regulatory System appears to be disordered in diabetic animals, and its function can be restored with NTX treatment. These studies suggest a fundamental role for the Opioid Growth Regulatory System in the pathobiology of diabetic complications and a need for studies to elucidate this role further.

1. Introduction

This review focuses on the Opioid Growth Regulatory System and its implications for the pathobiology of diabetes. It was presented, in part, as the Lorenz. E Zimmerman Tribute Lecture at the symposium in Dr. Zimmerman's honor jointly sponsored by the American Academy of Ophthalmology and the American Association of Ophthalmic Oncologists and Pathologists, Chicago, Illinois, October 19, 2014. Dr. Zimmerman was the founder of modern ophthalmic pathology having served at the Armed Forces Institute of Pathology for 52 years. He was mentor to many practicing ophthalmic pathologists and the recipient of numerous national and international honors. He died April 6, 2013, at the age of 92.

2. The Opioid Growth Regulatory System

2.1. System Overview. This review highlights the Opioid Growth Regulatory System. In particular, it emphasizes its implications for the pathobiology of diabetic complications including impaired wound healing, abnormal corneal sensitivity, and dry eye. Figure 1 highlights two of the main and opposing characters in this story: the naturally occurring opioid growth factor (OGF), [Met^5]-enkephalin, and its pharmacologic antagonist, naltrexone (NTX). This figure is the basis for the title of this review.

2.2. Roles of Endogenous Opioids. There are many endogenous opioids. They bind to specific receptors and they perform various biologic functions including analgesia, cardiovascular control, respiration, behavior, learning and memory, emotion, and cell division and growth. This review focuses on the latter function of regulation of growth and cell division by the Opioid Growth Regulatory System. This system, also called the OGF-OGFr axis, is comprised of two major components: opioid growth factor (OGF) itself and its specific "opioid growth factor receptor" (OGFr).

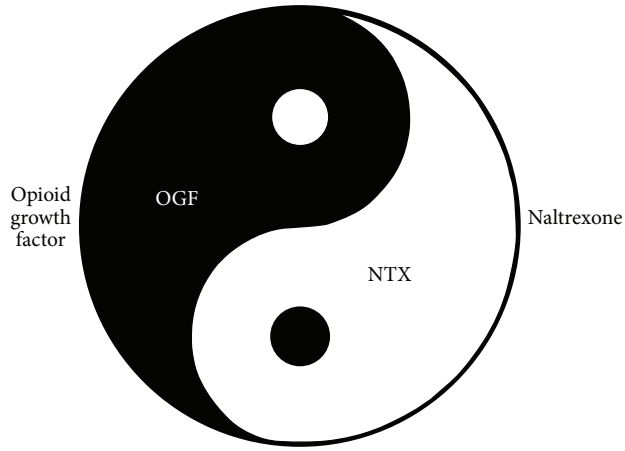

Figure 1: The figure illustrates the two opposing "Yin and Yang" entities: opioid growth factor (OGF), [Met^5]-enkephalin, which initiates downregulation of cell division, and naltrexone (NTX), which blocks the receptor (OGFr) for OGF resulting in upregulation of cell division and facilitation of epithelial wound healing.

2.3. Opioid Growth Factor and Its Receptor. Opioid growth factor, chemically [Met^5]-enkephalin, is a naturally occurring opioid. It is a pentapeptide with the sequence Tyr-Gly-Gly-Phe-Met. Its action is to depress cell division when bound to the other key component of the Opioid Growth Regulatory System, the specific opioid receptor for OGF, not surprisingly termed OGFr. OGF is potent and reversible and is species and tissue nonspecific.

The OGFr has been cloned and sequenced in the human, rat, and mouse. It has no resemblance to classic opioid receptors. Its specific gene locus is known. OGF bound to this specific receptor is the only such opioid that has an effect on cell division.

OGF is tonically produced so that usually its level in tissues is neither maximized nor minimized. As a result of this characteristic, manipulation of the Opioid Growth Regulatory System, either by the addition of exogenous OGF or by blocking its receptor, can decrease or increase cell division, respectively.

OGF usually is produced in an autocrine or paracrine manner, meaning that it is manufactured by the cells that will be modulated by it or by their neighbors. Nevertheless, systemic levels may be of importance for diabetic complications [1, 2]. It specifically targets cell proliferation.

The Opioid Growth Regulatory System is truly an ancient cellular regulatory mechanism that has been conserved from bacteria to humans [3, 4]. This system can modulate growth and development in embryologic, normally dividing, healing, and even neoplastic tissues (basically, any cell that has the potential to divide). The authors' observations suggest that it does not "overdrive" cell division in tissues that have attained contact inhibition of cell division. It does not alter apoptosis, necrosis, or differentiation.

2.4. Opioid Growth Regulatory System Mechanism of Action. When OGF is bound to its specific receptor, OGFr, cell division is suppressed.

Impact of OGF-OGFr Axis Manipulation on Cell Division

OGF = negative/inhibitory growth factor

NTX blocks OGFr = stimulates growth

$\uparrow$ OGF-OGFr $\longrightarrow$ $\downarrow$ cell replication

OGF- $\uparrow$ OGFr $\longrightarrow$ $\downarrow$ cell replication

$\downarrow$ OGF-OGFr $\longrightarrow$ $\uparrow$ cell replication

OGF- $\downarrow$ OGFr $\longrightarrow$ $\uparrow$ cell replication

OGF $\neq$ OGFr $\longrightarrow$ $\uparrow$ cell replication

$\uparrow$

Opioid antagonist (NTX)

Figure 2: Ways in which the relationship between OGF, OGFr, and NTX can be manipulated to impact cell division.

Figure 2 demonstrates several ways in which the relationship between OGF and its receptor can be manipulated to regulate cell division. For example, addition of exogenous OGF or an increase in the number of its receptors downregulates cell division. Conversely, one can increase cell division by decreasing the interaction of OGF with its receptor, either by decreasing the production of OGF or its receptor or by utilizing a blocking agent, like the strong opioid antagonist, naltrexone (NTX), to directly block OGF-OGFr interaction.

The presence of the Opioid Growth Regulatory System has been demonstrated in the corneal epithelium of all vertebrate orders including mammals, birds, reptiles, amphibians, and fish, some of which are demonstrated in Figure 3 [5].

Over the past 25 years, the authors' research team has delineated the role of the Opioid Growth Regulatory System in the homeostasis and healing of ocular tissues. More recently, as will be discussed shortly, it has been shown to play a role in the pathobiology of diabetic ocular complications, such as depressed epithelial wound healing, abnormal

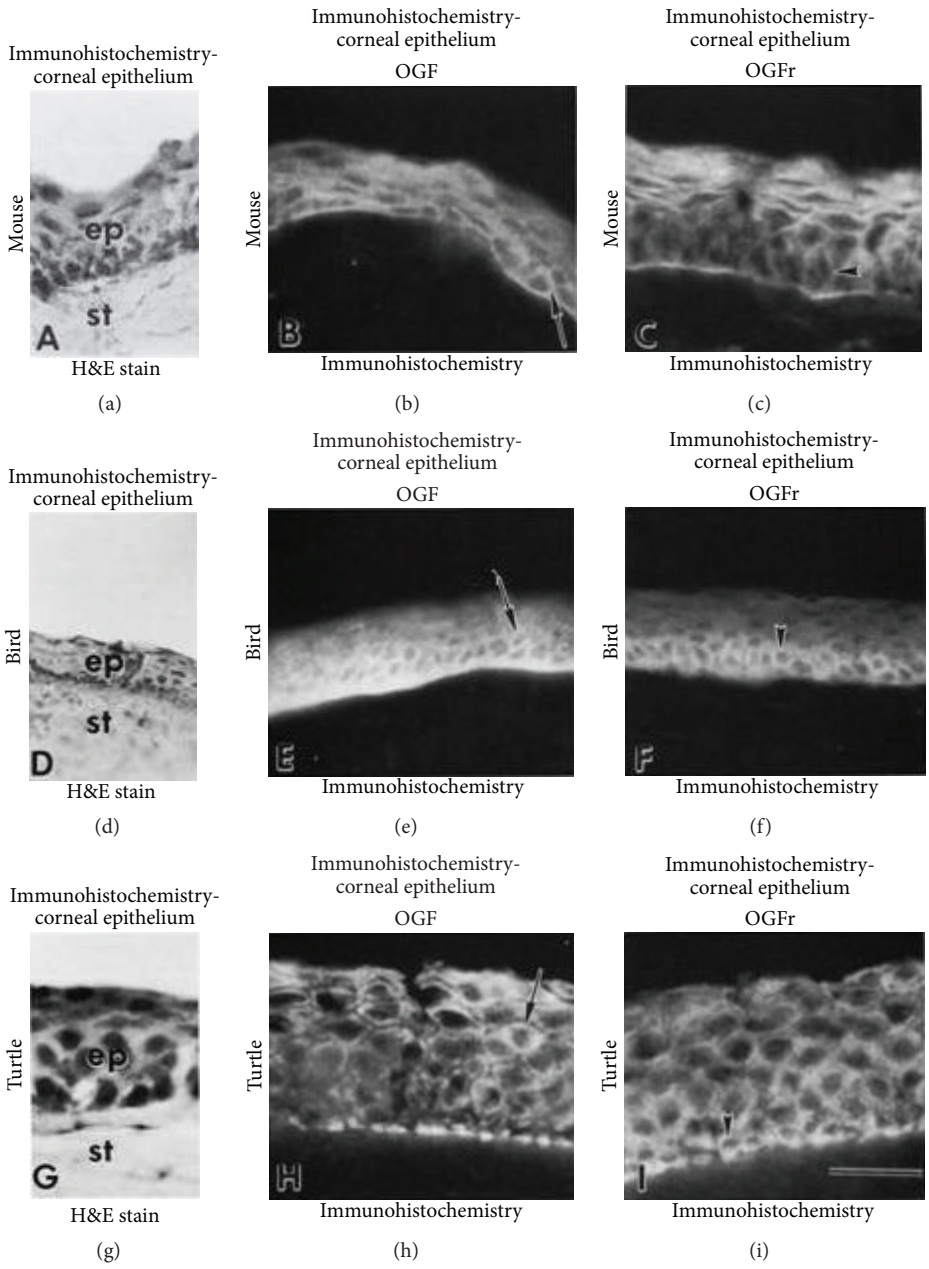

Figure 3: Immunohistochemistry for OGF and OGFr in mouse (a–c), bird (d–f), and turtle (g–i) corneal epithelium using brightfield (a, d, g) or indirect (b, c, e, f, h, i) immunofluorescence. Insets (a, d, g) are stained with hematoxylin and eosin and demonstrate the corneal epithelium (ep) containing basal and suprabasal cells with the underlying stroma (st). Immunofluorescence microscopy of tissue stained with anti-[Met^5]-enkephalin (OGF) IgG (b, e, h) demonstrates OGF in the epithelial cortical cytoplasm of basal and suprabasal cells (arrows). Immunofluorescence of tissues stained with OGFr IgG (arrowheads in c, f, i) (derived from [5]).

corneal sensitivity, and dry eye, and in the nonocular complication of delayed healing of diabetic cutaneous wounds.

3. Corneal Epithelial Growth Regulation

In the corneal epithelium, OGF appears to be produced in an autocrine manner. For example, immunohistochemical examination of the corneal epithelium in the peripheral cornea, limbus, and conjunctiva has demonstrated the presence of preproenkephalin, the precursor to OGF, within the corneal epithelium in these regions thereby supporting the autocrine production of OGF by the corneal epithelial cells [6].

3.1. Homeostatic Corneal Epithelium: Nonhuman. As seen in Figure 4, corneal explants in culture demonstrate that

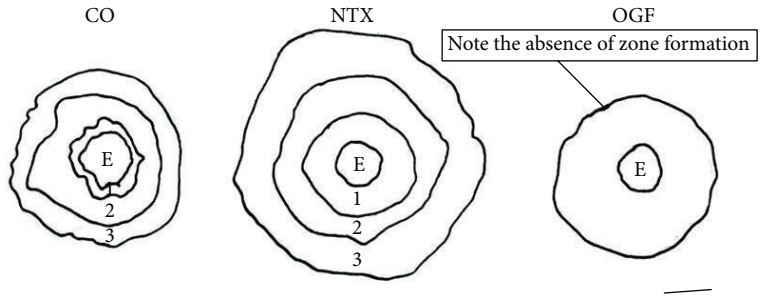

Figure 4: Outgrowths of corneal epithelium from corneal explants in organ culture from camera lucida drawings. CO: control; NTX: naltrexone supplemented culture media; OGF: opioid growth factor supplemented media; E: explant; numbers indicate zones of cellular outgrowth from the explant with zones 1 and 3 having active DNA synthesis and mitotic activity and zone 2 lacking DNA synthesis and mitotic activity. Note: explants exposed to media supplemented with OGF lack defined zone formation (derived from [7]).

the Opioid Growth Regulatory System modulates the outgrowth of homeostatic corneal epithelium, with exogenous OGF retarding and disorganizing the outgrowth and cell division of the epithelium and NTX accelerating outgrowth in reference to control explants without altering the normal outgrowth pattern [7, 8].

If the Opioid Growth Regulatory System can modulate corneal epithelial migration and cell division in tissue culture, what is its impact on homeostatic corneal epithelium *in vivo*? Figure 5 documents the ability of treatment with OGF to suppress DNA synthesis in the cornea of the living rat. Conversely, NTX treatment significantly increases DNA synthesis [8]. (Please note that all findings or data cited in this review are significant at a minimum of $P < 0.05$, but, for the sake of brevity, no specific significance values will be presented except as cited in figures and their captions.)

3.2. Epithelial Wound Healing: Nonhuman. If blockade of the Opioid Growth Regulatory System positively impacts epithelial outgrowth in tissue and organ culture and increases DNA synthesis *in vivo,* how would it impact corneal epithelial wound healing? Indeed, treatment with either systemic or topical NTX results in an increased rate of rat corneal epithelial wound healing [9–11]. As illustrated in Figure 6, either intraperitoneal or topical NTX significantly increases the rate of reepithelialization of standardized rat corneal epithelial wounds. Similarly, rabbit corneal epithelial wound healing also is increased by blockade of the Opioid Growth Regulatory System by topical NTX [9–11].

3.3. Gene Transfer and OGFr. Using the "Gene Gun," one can specifically determine the role of the interaction of OGF and its receptor (OGFr) in regulating epithelial wound healing by delivering sense or antisense OGFr cDNA into corneal epithelial cells (Figure 7) [12, 13]. Sense cDNA increases OGFr production and antisense suppresses OGFr production. Overexpression of OGFr results in delayed wound healing of rat corneal epithelial abrasions and suppression of OGFr production using antisense cDNA results in expedited wound healing.

3.4. Lack of Toxic Effects of NTX Treatment. Is the increased corneal epithelial wound healing that is achieved through manipulation of the Opioid Growth Regulatory System accompanied by proliferative abnormalities in the epithelium? In order to answer this question, animals were treated *in vivo* for one week with NTX [8, 14]. Figure 8 demonstrates that DNA synthetic cells increased by 69–85% in response to NTX treatment. Epithelial thickness also increased by 8 to 38%. Cellular packing density was increased; however, no toxicity or proliferative pathology was seen. Rather, NTX treatment accelerates normal homeostatic processes. There was negligible apoptosis or necrosis.

3.5. Healing Corneal Epithelium: Human. Just as the Opioid Growth Regulatory System regulates epithelial wound healing in animals, studies of organ cultured human corneas subjected to epithelial wounds demonstrated its impact on human epithelial wound healing. Figure 9 illustrates accelerated human corneal epithelial healing of organ cultured corneas grown in culture medium supplemented with 10^{-6} M NTX. Conversely, supplementation of culture medium with OGF suppresses epithelial wound healing (Figure 9) [1].

Finally, OGF and NTX impact cultured human corneal DNA synthesis as one might anticipate with increased synthesis resulting from NTX treatment and DNA synthesis suppression from OGF supplementation.

4. Diabetes and the Opioid Growth Regulatory System

So what does all this have to do with diabetes?

4.1. Background. Diabetes is the leading cause of blindness among working-age adults in the United States [15]. In 2012, 29.1 million Americans or 9.3% of the population had diabetes. The prevalence of diabetes rises to 25.9% of American seniors [16].

Among the complications of diabetes is keratopathy. Both type 1 diabetes and type 2 diabetes are associated with keratopathy that is reflected in delayed corneal epithelial

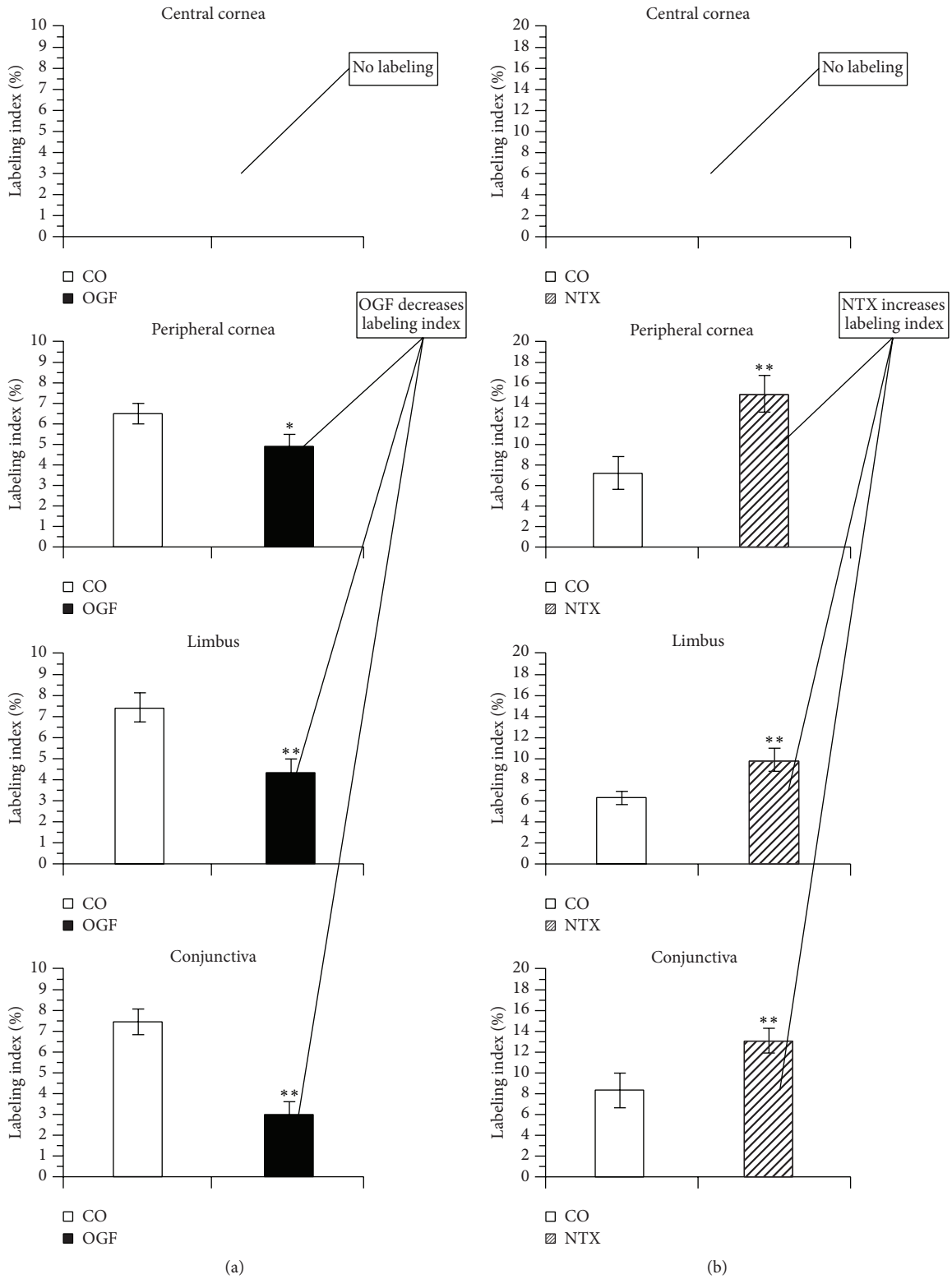

FIGURE 5: *In vivo* rat cornea: effects of OGF and NTX. Labeling index of basal corneal epithelium in the central cornea, peripheral cornea, limbus, and conjunctiva. Left series of histograms: OGF decreases labeling index of homeostatic rat corneal epithelium. Right series of histograms: NTX increases labeling index of homeostatic rat corneal epithelium. Note: neither OGF nor NTX has any effect on the central corneal epithelium (right and left, top graphs), which is postmitotic. $^{*}P < 0.05$ or $^{**}P < 0.01$ (derived from [8]).

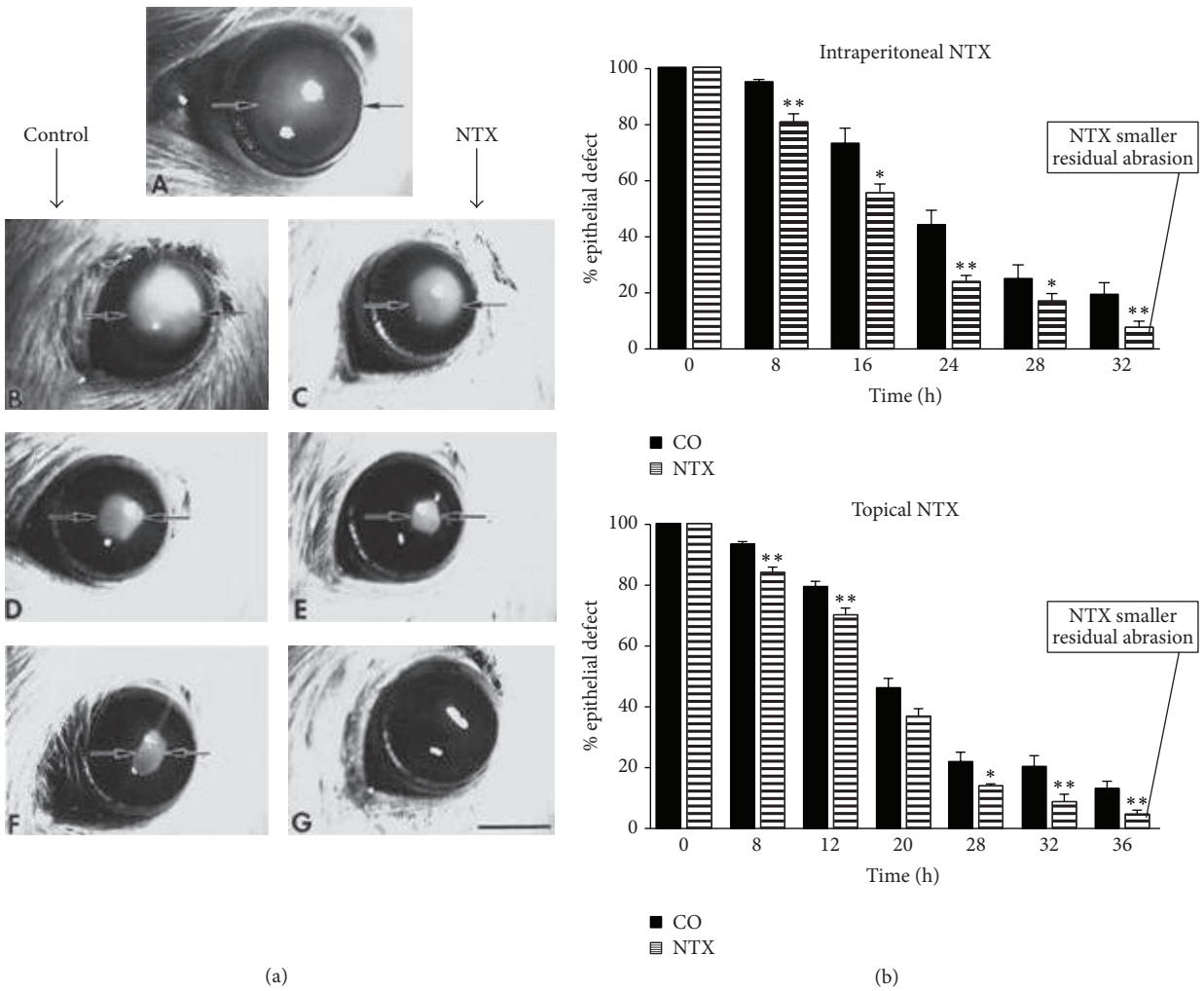

Figure 6: Rat cornea wound healing. (a) Photographs of rat corneas with 4 mm central abrasions stained with fluorescein immediately (A) or at 16 h (B, C), 24 h (D, E), or 32 h (F, G) after wounding and treated either with 30 mg/kg intraperitoneal injections of NTX (C, E, G) or with an equivalent volume of vehicle (B, D, F). (b) Histograms illustrate that both intraperitoneal injections of 30 mg/kg NTX or topical 10^{-6} M NTX eyedrops four times daily expedite rat corneal epithelial wound healing (derived from [10]). $^{*}P < 0.05$ and $^{**}P < 0.01$.

wound healing [17–19], abnormal corneal sensitivity [18–22], and dry eye [20, 21, 23, 24]. Unfortunately, none of the current treatments for these complications is uniformly effective [17].

Elevated levels of OGF, [Met^5]-enkephalin, have been found in the plasma of diabetic patients [25–27]. Elevated OGF levels also have been found in genetically obese diabetic (db/db) mice, which are used as a model for type 2 diabetes [28–30]. Moreover, OGF and OGFr have been found in the corneal epithelium in diabetic animals [31]. It was postulated that abnormalities of opioid regulation could contribute to the complications of diabetes and that blockade of the Opioid Growth Regulatory System by NTX might reverse or ameliorate these complications.

The relevance of the Opioid Growth Regulatory System to the following diabetic corneal complications: delayed epithelial wound healing, abnormal corneal sensitivity, and dry eye will be discussed separately.

4.2. Diabetic Keratopathy. During the course of their disease, seventy percent of diabetics will suffer from diabetic keratopathy, which includes recurrent erosion, delayed wound healing, edema, and even ulcers [32–34]. These complications may occur spontaneously [35] or follow specific insults, such as ocular surgery [36–39].

Immunocytochemistry confirms the presence of OGF and OGFr in the corneal epithelium of diabetic animals.

In order to determine whether blockade of the Opioid Growth Regulatory System would improve epithelial wound healing in diabetes, standardized corneal epithelial wounds were produced in rats after four weeks of induced diabetes. Treatment with intraperitoneal NTX twice daily resulted in a marked increase in the rate of corneal reepithelialization compared to untreated control animals [31]. Figure 10 illustrates the impact of NTX treatment on the rate of corneal epithelial healing in untreated diabetic rats. Untreated

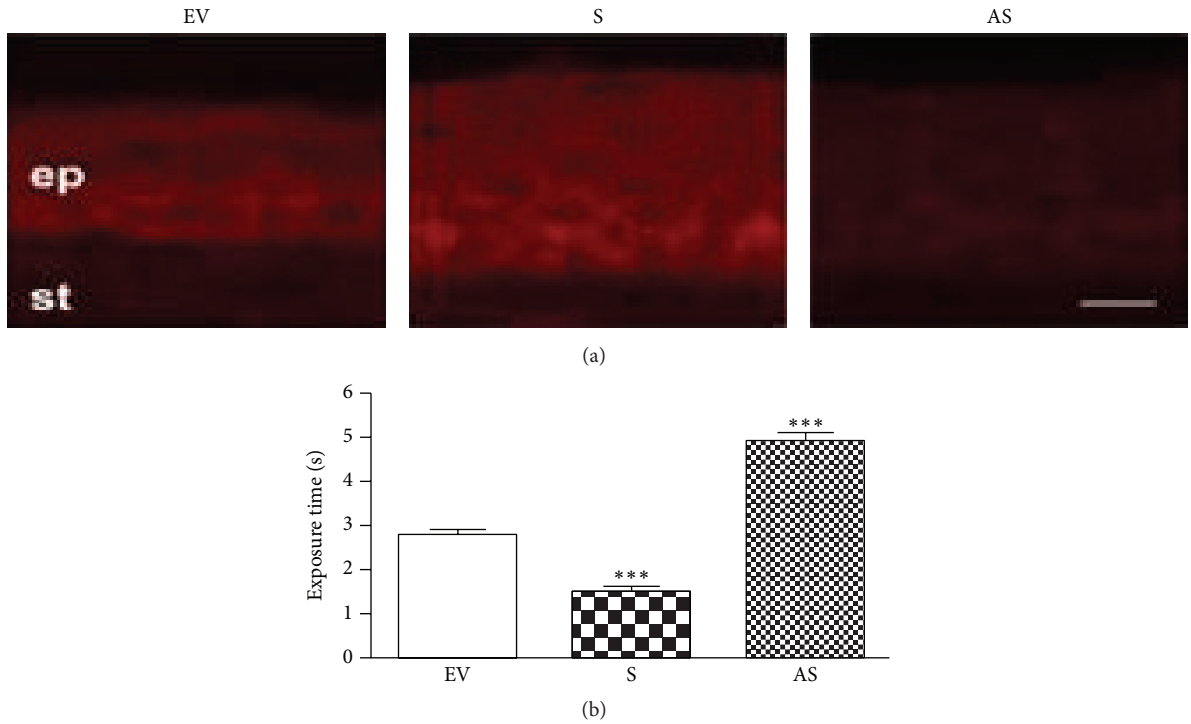

Figure 7: OGF-OGFr and molecular interactions: Gene Gun, Helios® Gene Gun System (Bio-Rad), used for particle-mediated gene transfer, which successfully delivers sense and antisense OGFr cDNA into corneal epithelial cells. (a) Fluorescence photomicrographs of immunohistochemical staining of peripheral cornea for OGFr expression. Sense (S) results in overexpression of OGFr. Antisense (AS) results in diminished expression of OGFr. Empty vector (EV) has no effect. (b) Histograms illustrate immunoreactivity response to particle-mediated transfection with empty vector (EV), sense (S), or antisense (AS). $^{***}P < 0.001$. Note: the y-axis is exposure time so an increased exposure time required for detection of the OGFr indicates a decreased amount of OGFr present in the sample (derived from [13]). All stained with OGFr antibodies.

diabetic animals healed at a rate that was significantly worse than that in normal controls. On the other hand, diabetic NTX-treated animals healed at a rate equal to that of the normal controls. Therefore, having demonstrated the ability of NTX to increase cell proliferation, it is not surprising that NTX treatment increased DNA synthesis in unwounded diabetic rat corneas 4-fold in the basal epithelium of the peripheral cornea, 3.5-fold in the limbal region, and 8-fold in the conjunctiva compared to control animals [31].

Does glucose control improve epithelial wound healing in diabetic rats and if so does NTX have an insulin-like effect on corneal epithelial wound healing?

In order to answer these questions, corneal epithelial wound healing in untreated diabetic rats was compared to that in animals treated with insulin minipumps [40]. At 40 hrs after wounding, untreated diabetic (DB) rats had significantly larger residual epithelial defects than the controls (either nondiabetic or DB-insulin-treated rats). This and other studies demonstrated clearly that intensive therapy with insulin, leading to normoglycemia in rats with diabetes, does prevent the delay in wound healing of ocular surface epithelium observed in poorly controlled diabetic animals.

Given that systemic control of diabetes facilitates corneal epithelial wound healing, does topical insulin have an effect independent of systemic glucose control?

In rats that have been diabetic for 9 or 11 weeks, topical insulin was administered four times daily for 7 days to wounded corneas. Diabetic animals treated with vehicle alone had wounds that were 35% larger than those in healthy vehicle-treated animals [40, 41]. Topical insulin treatment resulted in epithelial wounds that were 19% to 60% smaller than diabetic vehicle-treated ones. There was no insulin effect on healthy rat epithelium, and there was no effect on corneal thickness, IOP, apoptosis, or serum glucose levels. Thus, topical insulin treatment is effective in reversing the delayed epithelial wound healing characteristic of diabetic animals.

What is the effect of NTX treatment on corneal epithelial wound healing in diabetic animals?

In preparation for testing NTX treatment for epithelial defects, a toxicity study of topical NTX was performed in insulin-controlled diabetic rats (Figure 11) [42]. There was no difference from normal rats or insulin-treated diabetic controls in IOP, corneal thickness, endothelial cell number, or epithelial apoptosis, necrosis, or organization. There was no overt toxicity of NTX over a 10,000-fold range of dosage.

Similarly, topical NTX proved as effective as intraperitoneal treatment for more rapidly healing epithelial defects in diabetic animals, and topical insulin was equally effective and safe as topical NTX for this purpose [40, 41, 43].

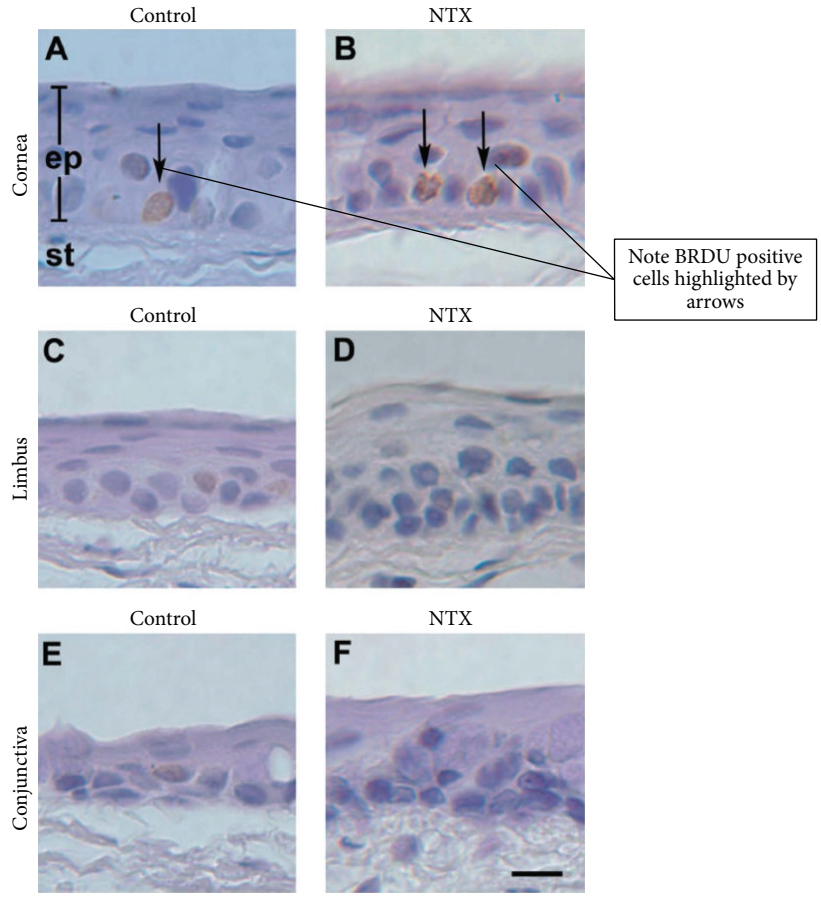

Figure 8: Impact of chronic NTX on homeostatic corneal epithelium. Seven-day treatment with systemic NTX. Rate of DNA synthesis determined using BrdU. Arrows are BrdU-positive, DNA synthetic cells. DNA synthetic cells increased by 69–85% following NTX treatment. Epithelial thickness increased by 8 to 38%. There was increased packing density. Nevertheless, no toxicity or proliferative pathology was seen. Rather, NTX treatment accelerates normal homeostatic processes, but there is negligible apoptosis or necrosis. st: stroma; ep: epithelium. Bar = 12 μm (derived from [14]).

Combining topical insulin and topical NTX was not more effective than either one used independently [40, 41, 43, 44]. In short, there is a possibility that insulin and NTX have their effect through similar mechanisms in diabetic animals or that each medication has the potential to maximize epithelial wound healing in these animals, leaving no opportunity for further increase by the complementary modality. Furthermore, insulin has no effect on epithelial proliferation in normal animals, and NTX has no impact on blood glucose levels in diabetic animals.

These data were compared to that involving the healing of corneal epithelial wounds in untreated or systemic insulin-treated diabetic rats given topical NTX (Figure 12). In both treatment groups, topical NTX significantly increased the rate of epithelial wound healing in contrast to the situation when insulin and NTX are combined in topical administration. One possible explanation for the difference in results obtained relative to the route of insulin administration (systemic versus topical) may be the inability of systemic insulin to reach the tear film in a concentration equivalent to that obtained with topical administration.

One should note that although NTX has been discussed relative to induced type 1 diabetes, it also is effective in facilitating corneal epithelial wound healing in obese db/db mice with type 2 diabetes on a genetic basis [45].

The potential toxicity of NTX applied topically four times daily for 7 days in concentrations of 10^{-3} to 10^{-7} M was evaluated *in vivo* in intact and abraded corneas of insulin controlled or uncontrolled diabetic rats [44]. Ocular surface morphology, intraocular pressure, corneal thickness, and corneal sensitivity were evaluated. Histopathologic studies were performed for apoptosis, necrosis, and endothelial cell counts. No toxicity from NTX treatment was found.

In summary, in diabetic animals, topical NTX restores corneal epithelial wound healing to levels comparable to systemically or topically insulin-treated animals without apparent epithelial toxicity.

4.3. Diabetic Corneal Neuropathy. Diabetic corneal neuropathy, particularly as assessed by confocal microscopy, correlates with peripheral neuropathy [46–52]. Corneal aesthesiometry (measuring corneal sensitivity to touch using

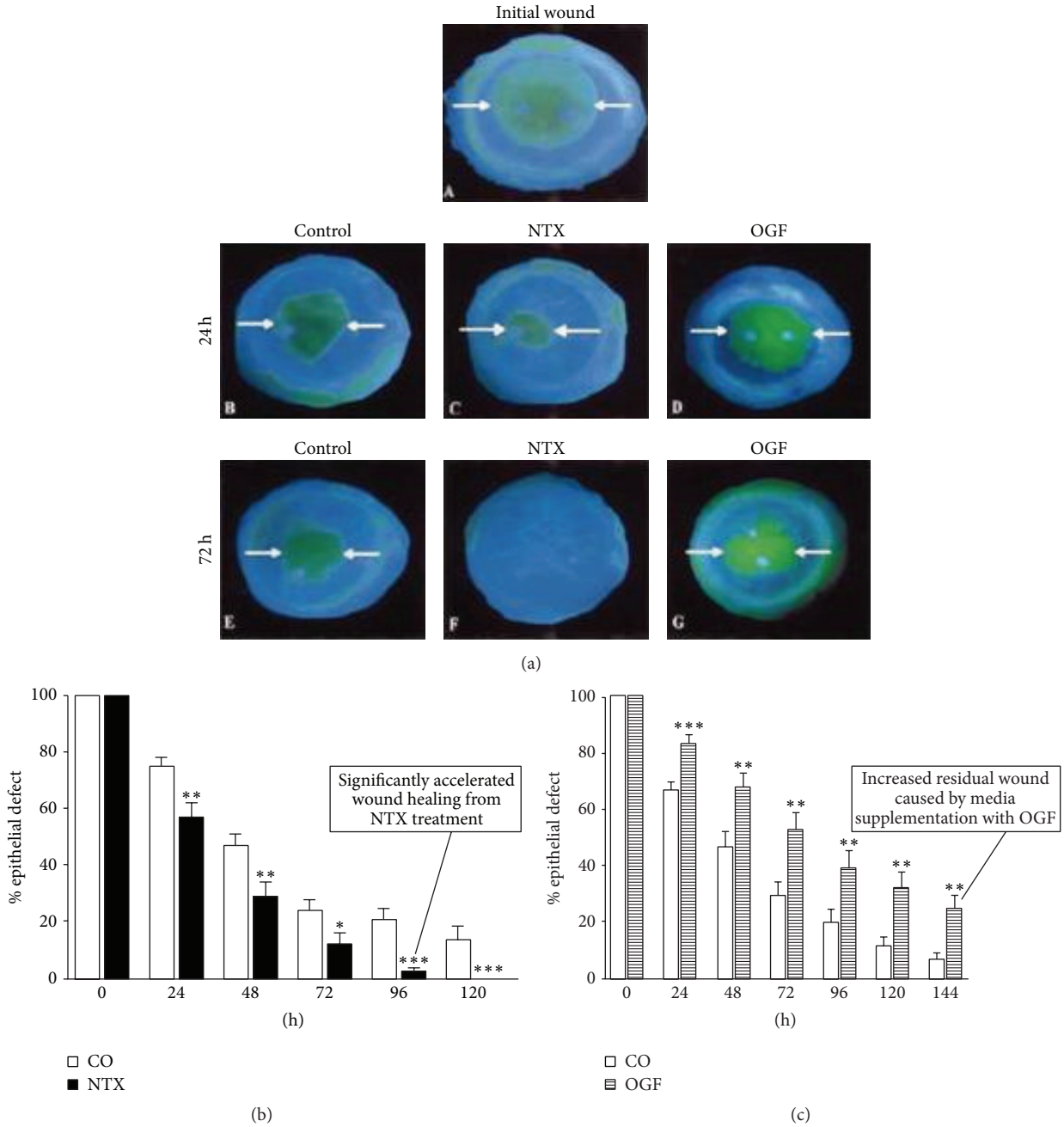

Figure 9: Human corneas in organ culture. (a) Photographs of abraded human corneas in organ culture at time of initial wounding and 24 and 72 hrs after wounding. Media supplemented with either sterile water (control) (B, E), 10^{-6} M NTX (C, F), or 10^{-6} M OGF (D, G). Note the more rapid healing response in NTX supplemented medium, compared to control, and decreased rate of wound healing with OGF medium supplementation. Arrows indicate the margins of the residual wounds. (b) Histograms illustrate the accelerated rate of epithelial wound healing in response to NTX culture medium supplementation. Overall, the healing of epithelial wounded human corneas cultured in medium supplemented with 10^{-6} M NTX was accelerated by 21% to 89% during the period of 24 to 96 hours. (c) Supplementation of culture medium with OGF suppresses epithelial wound healing. Histogram of residual epithelial defect in organ cultured human corneas following 8 mm epithelial wounds. Medium was supplemented with either sterile water (CO) or 10^{-6} M OGF. 24% to 260% more defects present in OGF-treated corneas at day 7. Data are expressed as means ± SEM. Significantly different from controls at $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$ (derived from [1]).

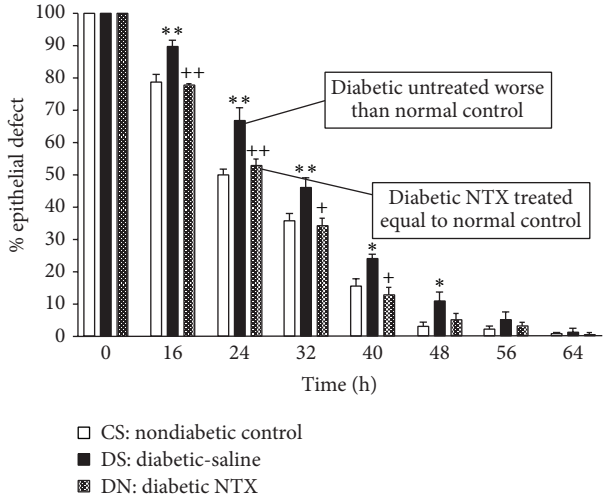

Figure 10: Naltrexone treatment normalizes epithelial wound healing in diabetic rats. Residual epithelial defect up to 64 h after creation of a 5 mm corneal abrasion. DS (untreated diabetic) had significantly larger residual epithelial defects than the CS (nondiabetic) or DN (NTX-treated diabetic rats). Therefore, NTX treatment prevents the delay in wound healing of ocular surface epithelium observed in poorly controlled diabetic animals. Data are expressed as means ± SEM and represent the residual of the original defects presented as a percentage of the original wounds. Significant difference between the DS and CS groups, $^{*}P < 0.05$ or $^{**}P < 0.01$. Significant difference between the DS and DN groups, $^{+}P < 0.05$ or $^{++}P < 0.01$ (derived from [31]).

progressively stiffer filaments, which are von Frey hairs) also can be helpful in the clinical assessment of diabetic corneal neuropathy [20, 50, 53, 54]. Diabetic corneal neuropathy is accompanied by delayed epithelial wound healing as described previously [55]. Corneal nerve damage can be induced by obesity related to diet or to type 2 diabetes [56]. Moreover, diabetic corneal nerve injury is repairable as evidenced by the fact that corneal nerve regeneration has been demonstrated after simultaneous kidney and pancreas transplantation [57] and other therapies [58].

In order to evaluate the reversibility of corneal diabetic neuropathy, rats having eight weeks of induced diabetes were treated with 1 or 5 days of four times daily NTX at 10^{-5} M concentration (Figure 13) [42, 59]. Corneal sensitivity was restored to normal levels beginning one hour after termination of drug exposure and extending for at least 4 days thereafter. Conversely, control diabetic animals maintained sensitivity scores that were 1.5- to 2.0-fold less than both the normal and NTX-treated groups.

The NTX effect resulting in normalization of corneal sensitivity ended after 120 hrs, for animals treated for one day, and 192 hrs following discontinuation of a 5-day treatment period with four times daily NTX. At those respective time points, corneal sensitivity reverted to being 1.9-fold less than normal animals and comparable to control diabetic animals. Thus, the period of normalcy only can be attributed to NTX therapy.

NTX also is effective in restoring corneal sensitivity to normal levels in obese db/db type 2 diabetic mice [45].

In summary, topical NTX treatment restores corneal sensitivity to normal levels in both type 1 and type 2 diabetic rats. These findings implicate the Opioid Growth Regulatory System in the pathobiology of diabetic corneal neuropathy and are consistent with other studies cited above, which suggest that diabetic corneal neuropathy can be reversible.

4.4. Dry Eye. Dry eye is more common in diabetic patients and correlates with poor glycemic control [21, 23, 60]. Moreover, diabetic dry eye is more common in individuals with diabetic retinopathy of increased severity [61].

Apparently normal rats have periods during which there is a spontaneous decrease in tear production (Figure 14) [62]. It was determined that one drop of 10^{-5} M NTX restores tear production to normal levels for up to 48 hrs in such animals. Vehicle alone results in no improvement in tear secretion. If right and left eyes are compared after one drop of 10^{-5} M NTX in the right eye, there is no effect on the contralateral eye.

Conversely, neither one drop of 10^{-5} M NTX nor vehicle had any impact on tear production that already was at a normal level. There was no difference in corneal sensitivity during periods of normal or reduced tear production over a 20-fold difference in force using von Frey hairs, which are used to test for corneal sensitivity to touch [62].

Although NTX has no ability to raise tear production in rats with normal tear secretion, one drop of 10^{-5} M OGF, [Met^5]-enkephalin, significantly reduces tear production in rats with initially normal levels (Figure 15) [62].

Thus, NTX blockade of the Opioid Growth Regulatory System appears to have the ability to raise tear production to normal levels in nondiabetic rats having a period of depressed tear production. Conversely, OGF can depress tear production to subnormal levels even in nondiabetic rats. These data support the concept of Opioid Growth Regulatory System modulation of tear production even in nondiabetic rats.

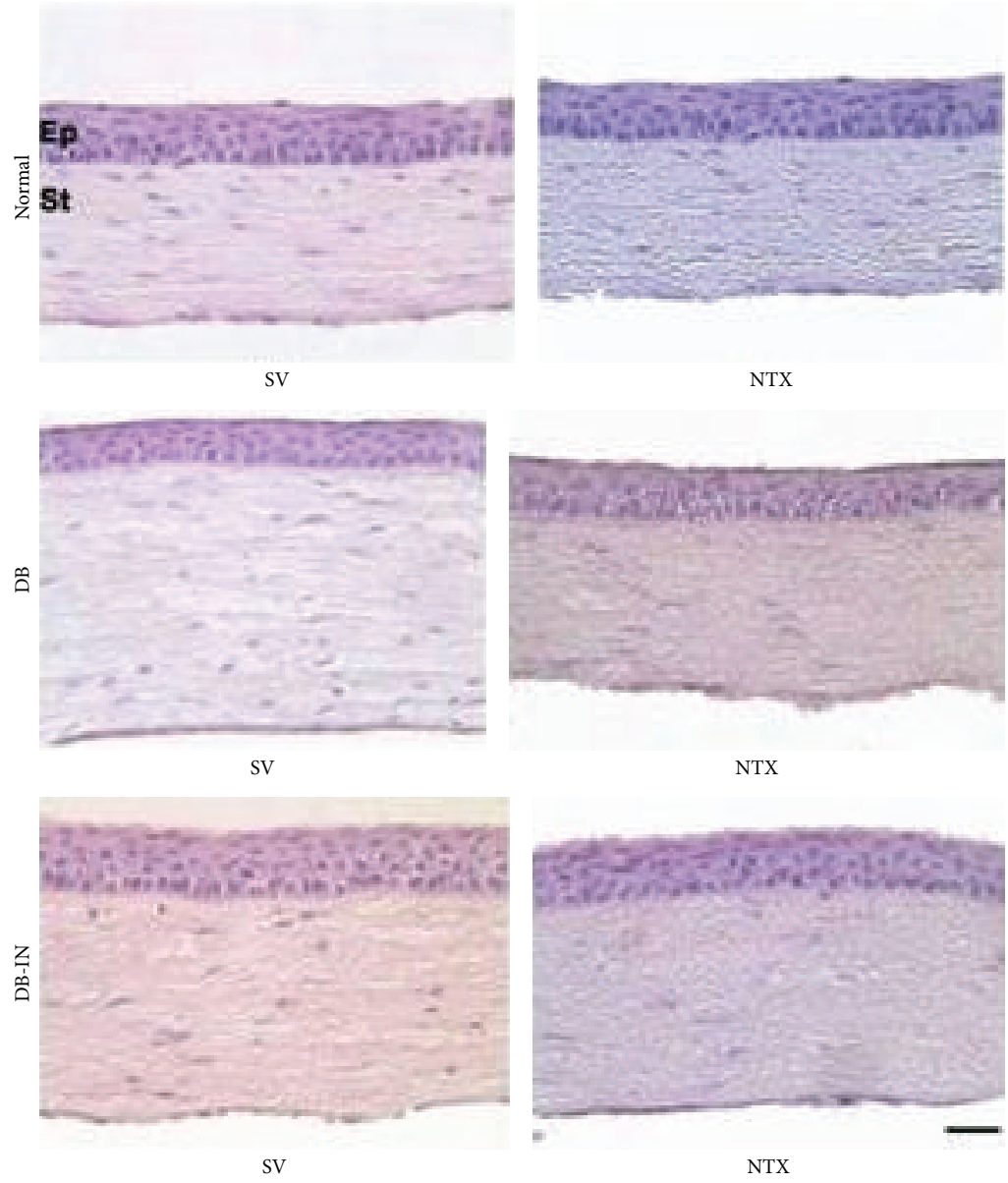

Figure 11: Toxicity evaluation of NTX-treated rat cornea. Corneal thickness, endothelial cell number, and evidence of epithelial apoptosis, necrosis, or organization were determined for topical 10^{-3} to 10^{-7} M NTX administered q.i.d. × 7 days to corneas of type 1 diabetic rats. Photomicrographs of histological sections of the rat peripheral cornea stained with hematoxylin and eosin from animals in normal, diabetic (DB), and diabetic-insulin (DB-IN) groups at 2 weeks after the conclusion of 7-day exposure (4 times daily) of 10^{3} M naltrexone (NTX) or sterile vehicle (SV). The morphology of the cells and tissues was similar between groups, and no pathologic changes were detected in corneas exposed to vehicle or 10^{3}–10^{7} M NTX (data not shown for treatment with 10^{-4}, 10^{-5}, 10^{-6}, or 10^{-7} M NTX). Ep: epithelium; St: stroma. Bar = 40 m (derived from [42]). These photomicrographs are of rat peripheral cornea treated with the topical 10^{-3} M NTX administered q.i.d. × 7 d or controls.

What is the impact of diabetes on tear production? Dry eye was evaluated in rats having type 1 diabetes of 8-week duration treated with four times daily topical NTX at 10^{-5} M concentration. Untreated diabetic rats had tear production reduced by 32% to 53% compared to normal or to NTX-treated animals. In contrast, diabetic rats treated with NTX had tear production similar to normal rats extending for at least 3 days following the termination of treatment. By 96 hours after termination of treatment, tear production had decreased again to 22% to 59% less than normal animals, thereby emphasizing how effective the previous NTX treatment had been.

Figure 16 illustrates tear production in wild-type or db/db type 2 diabetic mice given one drop of 10^{-5} M NTX (a) or only vehicle (b). Note the rise in tear production to normal levels with NTX treatment until about 72 hrs after treatment (a). There was no effect from vehicle alone (b) on abnormal tear production at all times tested [45].

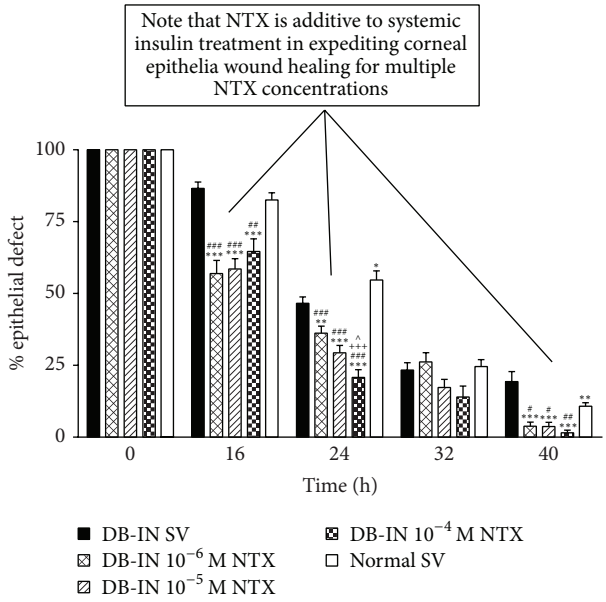

Figure 12: The figure compares the effectiveness of systemically administered insulin and topical NTX. Histograms demonstrate that topical NTX is additive to systemic insulin in facilitating epithelial wound healing in diabetic rats. Residual epithelial defects are presented as percentage of the original wound. Data expressed as means ± SEM. Significantly different from DB-IN rats receiving SV at $^{**}P < 0.01$ or $^{***}P < 0.001$ and from normal SV rats at $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, or $^{\#\#\#}P < 0.001$. At 24 hrs, the 10^{-4} M NTX DB-IN group differed from the 10^{-5} M NTX DB-IN group at $^{\wedge}P < 0.05$ and from the 10^{-6} M NTX DB-IN group at $^{+++}P < 0.001$ (derived from [44]).

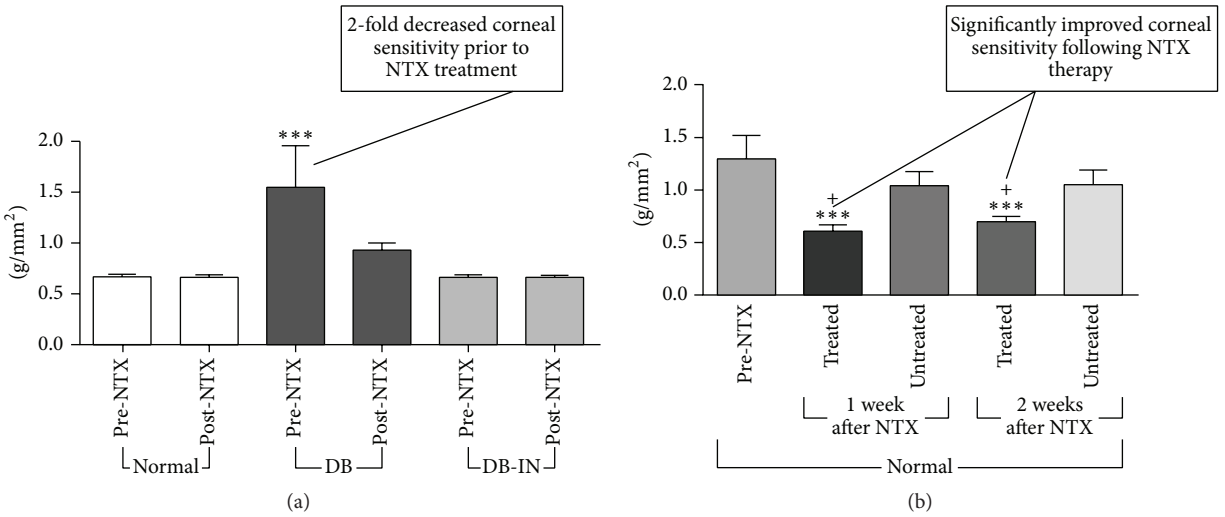

Figure 13: Impact of NTX treatment on corneal sensitivity. (a) Prior to NTX treatment: 2-fold decreased corneal sensitivity in the 8-week DB untreated diabetic rats compared to normal controls (increased force required to generate a response). (b) After NTX treatment, DB sensitivity returned to normal levels with a 2-fold and 1.5-fold increase in sensitivity, respectively, following NTX treatment. There was no effect on corneal sensitivity and there was no effect on normal or insulin treated diabetic (DB-In) rats. Note: in both histograms, higher bar is less sensitive. Values are means ± SEM. Significantly different from NTX at $^{***}P < 0.001$ and untreated corneas at $^{+}P < 0.05$ (derived from [42]). Height of histogram indicates force required for stimulus to be detected. Higher histogram indicates less sensitivity.

In summary, these findings support a role for Opioid Growth Regulatory System in the pathobiology of abnormal diabetic tear production in that NTX treatment restores tear production to normal levels for up to 3 days following discontinuation of the therapy in type 1 or type 2 diabetic rats.

4.5. Other Diabetic Complications: Cutaneous Ulcers. The research reported above demonstrates that the Opioid Growth Regulatory System is important in the pathobiology of diabetic ocular surface disease, in that manipulating the system through blockade of the OGFr with NTX restores

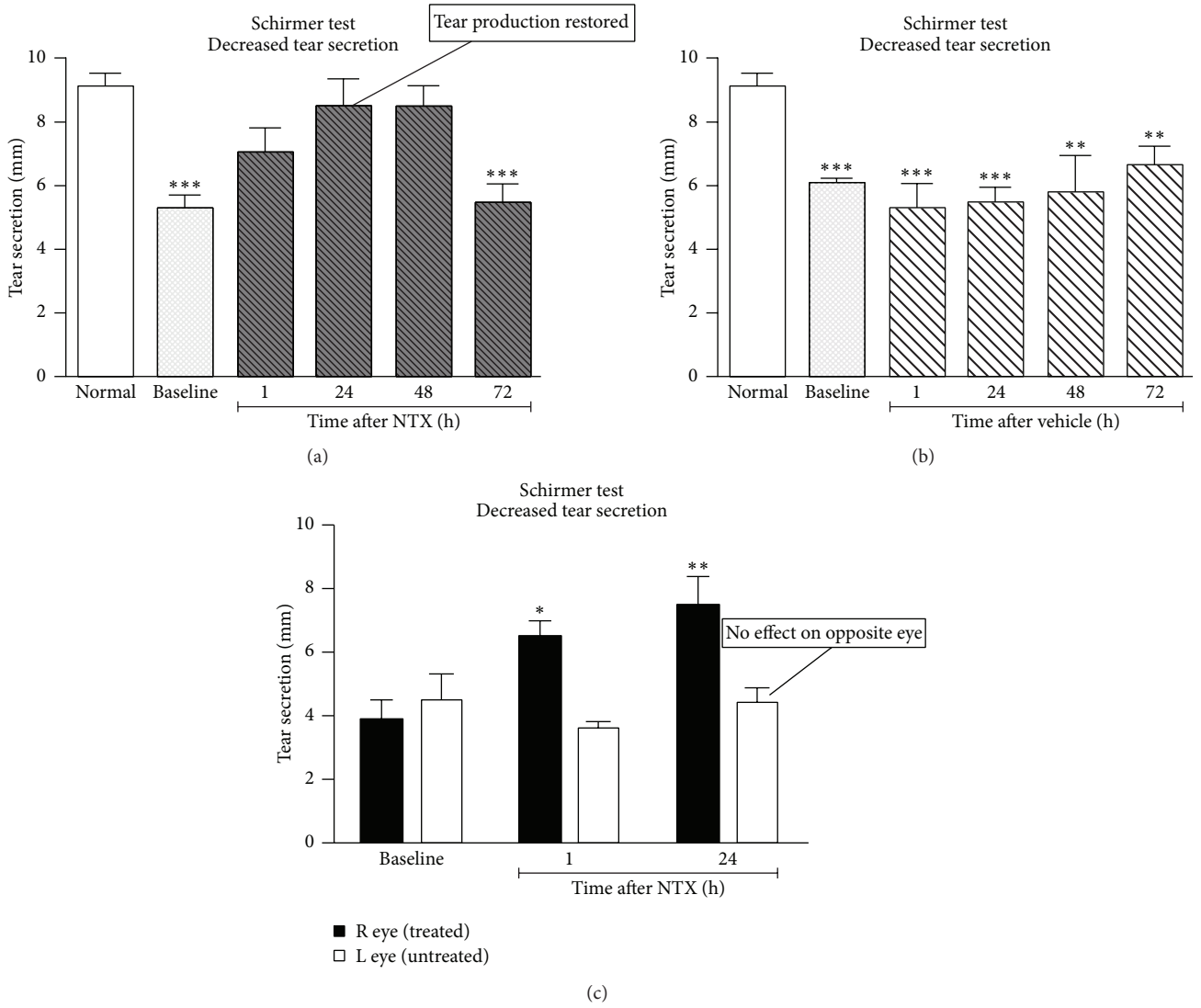

Figure 14: Impact of NTX treatment on tear secretion. (a) One drop of 10^{-5} M NTX results in tear levels returning to normal for up to 48 hr. (b) Vehicle alone results in no improvement in tear secretion. Significantly different from normal rats at $^{**}P < 0.01$ or $^{***}P < 0.001$. (c) Right and left eyes compared after one drop of 10^{-5} M NTX in the right eye. Significantly different from left eye at $^{*}P < 0.05$ and $^{**}P < 0.01$. Values represent means ± SEM (derived from Zagon et al., 2012 [62]).

corneal epithelial wound healing, ocular sensation, and tear production to normal levels in diabetic animals. Nevertheless, the question arises as to whether these findings are only of localized importance restricted to the ocular region or whether they are manifestations of a systemic abnormality of the Opioid Growth Regulatory System secondary to diabetes.

Delayed or incomplete healing of cutaneous wounds, particularly foot ulcers, is an important systemic diabetic complication. For example, diabetic foot ulcers are said to be one of the most costly and devastating complications of diabetes mellitus and affect 15% of diabetic patients during their lifetime [63]. Therefore, the impact of Opioid Growth Regulatory System blockade on cutaneous wound healing in diabetic rats was evaluated to test the effectiveness of NTX on this important and quantifiable indicator for the systemic complications of diabetes.

The following research was performed by Jessica Immonen, a graduate student then and now Doctor Immonen, at the Department of Anatomy at Rocky Mountain University of Health Professions, as the basis for her Masters and Doctorate degrees. Working with Drs. Zagon and McLaughlin, from our research team, Dr. Immonen evaluated the impact of NTX 10^{-4} M, NTX 10^{-5} M, or NTX 10^{-6} M in Sorenson's phosphate buffer, lubricant, moisturizing cream, or dimethylsulfoxide applied to the skin surface three times daily on DNA synthesis in the skin of normal or type 1 diabetic rats (Figure 17). She discovered that cutaneous DNA synthesis in unwounded normal rats was elevated by 43% to 132% in response to NTX in any of the four vehicles compared to normal baseline. NTX applied three times daily topically to dorsal skin of DB rats elevated labeling index (LI) by 103–147% in lubricant and by 85–89% in moisturizing cream.

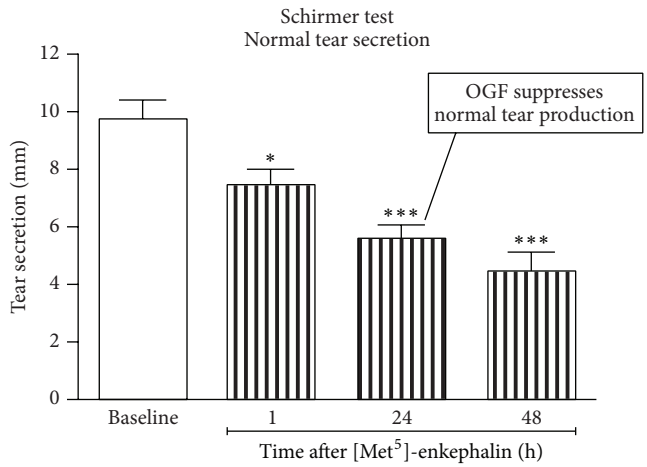

Figure 15: Impact of OGF treatment on tear secretion. One drop of 10^{-5} M OGF, [Met^5]-enkephalin, significantly reduces tear production in rats with initially normal levels. Values represent means ± SEM (derived from [62]). * refers to $P < 0.05$ and *** refers to $P < 0.001$.

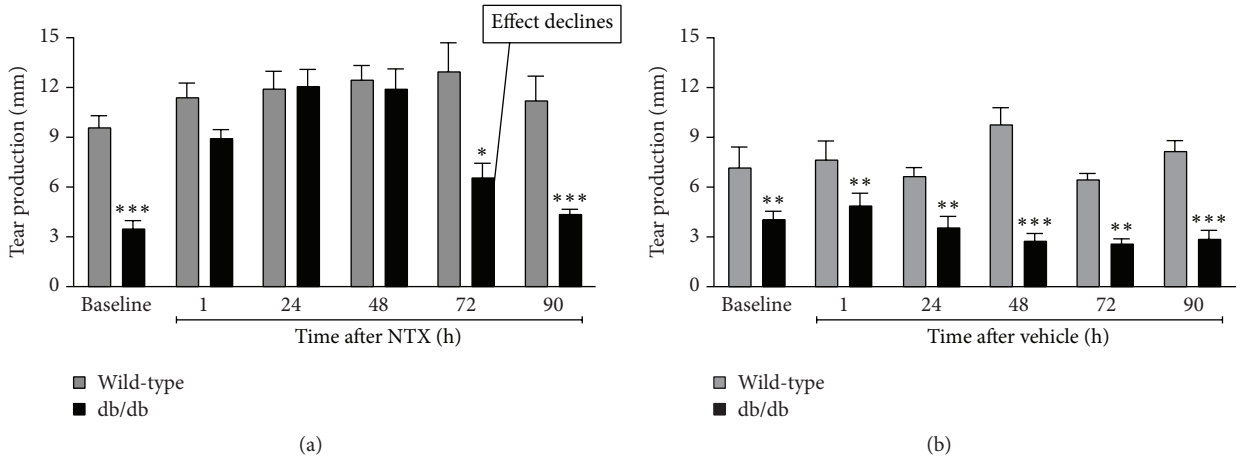

Figure 16: Tear production in wild-type or db/db mice given one drop of 10^{-5} M NTX (a) or only vehicle (b). (a) Note the rise in tear production to normal levels with NTX until about 72 hrs after treatment. No effect from vehicle alone (b) with abnormal tear production at all times tested. Ocular surface abnormalities related to type 2 diabetes are reversed by the opioid antagonist naltrexone. Significantly different from measurements for corresponding wild-type mice at each time point at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (derived from [45]).

Note the DNA synthesizing cells in the photomicrographs in Figure 17 [64].

A model of 6 mm full-thickness cutaneous wounds was used to investigate the healing response to one of the previously tested concentrations of NTX, 10^{-5} M, in either moisturizing cream (MCN) or vehicle alone (Figure 18). Within 3 days of treatment initiation, normal rats treated with once or three times daily NTX had wounds 30% and 11% smaller at the respective dosages than control animals. Diabetic animals treated with NTX in moisturizing cream had wounds 13% to 57% smaller than diabetic controls. There was no difference in skin histology between NTX-treated and control animals [64].

When normal (N) rats with standard skin wounds were treated three times daily with 10^{-5} M NTX in moisturizing cream, they healed 6–26% faster than normal control rats. Diabetic NTX-treated rats had wounds 62–89% smaller than diabetic controls (Figure 19) [65].

NTX appears to improve cutaneous wound healing, in part by stimulating angiogenesis. Diabetic control animals have delayed expression of fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF). Conversely, topical NTX stimulates expression of these angiogenic factors. Similar findings are noted for the expression of α-smooth muscle actin (SMA) in capillaries [65].

In summary, topical NTX accelerates cutaneous wound closure, at least in part, by stimulating expression of angiogenic factors within healing tissue of diabetic animals. Obviously, there is the potential for NTX treatment to have a significant impact on facilitating the initial closure of such wounds in diabetic patients.

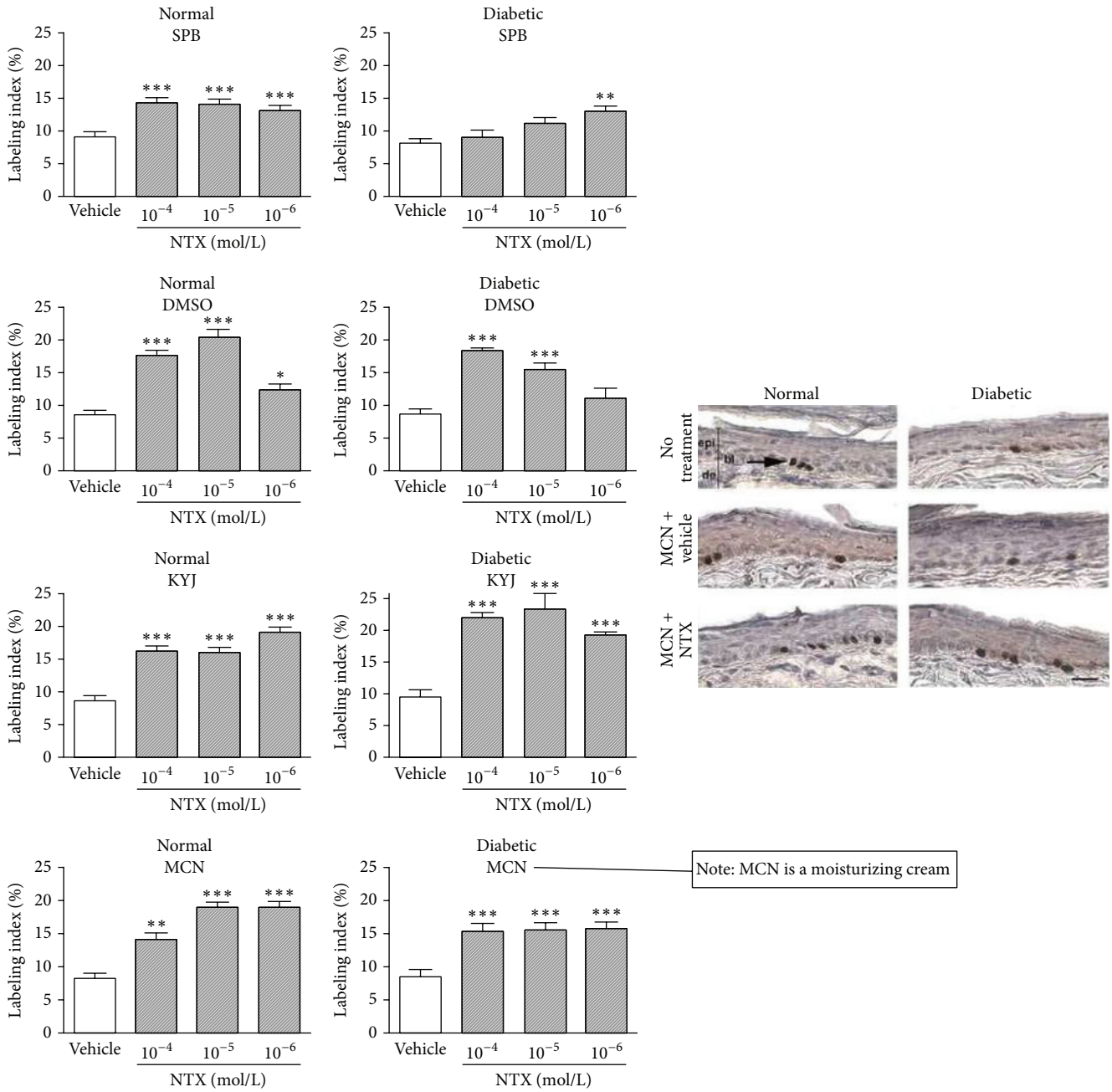

Figure 17: Effect of topical NTX in various vehicles on labeling index (LI) of skin epithelium. NTX was applied topically to the skin in type 1 diabetic rats or normal rats using NTX 10^{-4} M, NTX 10^{-5} M, or NTX 10^{-6} M in Sorenson's phosphate buffer, lubricant, moisturizing cream, or dimethylsulfoxide. NTX applied TID topically to dorsal skin of normal rats elevated LI by 43% to 132% in any of the four vehicles compared to normal baseline. NTX applied TID topically to dorsal skin of DB rats elevated LI by 103–147% in lubricant and by 85–89% in moisturizing cream. Photomicrographs demonstrate relative frequency of DNA synthesizing cells containing label. SPB: buffered saline; DMSO: dimethylsulfoxide; KYJ: sterile lubricating jelly; MCN: moisturizing cream (derived from [64]). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

NTX has a more pervasive impact on the overall process of wound healing beyond just wound closure. For example, birefringence of Sirius red-stained healing granulation tissue revealed increased collagen formation and maturation in NTX-treated animals (Figure 20) [66].

Finally, inadequate wound healing at 60 days after wounding in diabetic control animals is further demonstrated by reduced tensile strength in comparison to control nondiabetic animals or to NTX-treated diabetic wounds, which have tensile strength similar to normal controls [66].

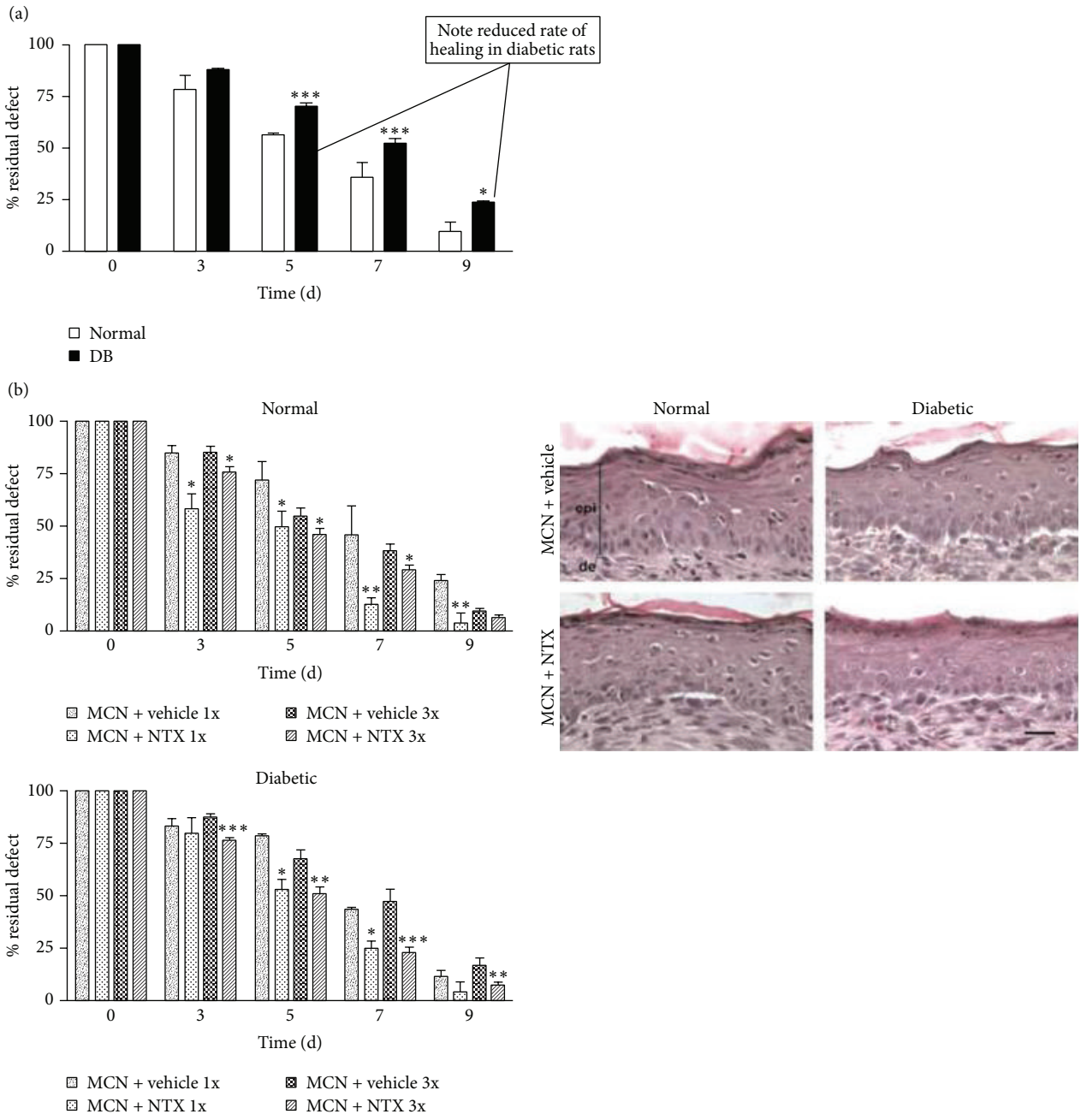

Figure 18: Effect of NTX treatment on wound healing rate of standardized skin wounds. Left: the impact of daily or three times daily NTX in moisturizing cream (MCN) on the healing rate of standard cutaneous wounds. Left (a): untreated diabetic rats had skin wounds that were 24%, 44%, and 132% greater than those of normal animals on days 5, 7, and 9, respectively, after wounding. Left (b, upper): within three days of a single or three times daily regimen of 10^{-5} M NTX in MCN, the wounded areas of normal rats were reduced by 30% and 11%, on the respective days, compared to other normal animals receiving MCN + vehicle. Left (b, lower): diabetic (DB) animals subjected to once daily application of MCN + NTX had a decreased wound size compared with DB rats exposed to vehicle, but the greatest effect was achieved with MCN + NTX given three times daily, which consistently accelerated wound closure, resulting in mean residual wounds that were reduced by 13% to 57% from DB rats treated with MCN + vehicle. Values represent means ± SEM. Significantly different from normal or MCN + vehicle at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Right: histology of skin comparing normal and DB rats receiving vehicle or NTX. The overall appearance is similar, without evidence of necrosis, and so forth. Nevertheless, epithelial thickness in normal rats ($24.6 \pm 1.6\,\mu$m) was 44% greater than in DB rats receiving vehicle. Normal animals treated with NTX, as well as DB rats subjected to NTX, did not differ in the thickness of the epithelium relative to normal animals receiving vehicle (derived from [64]).

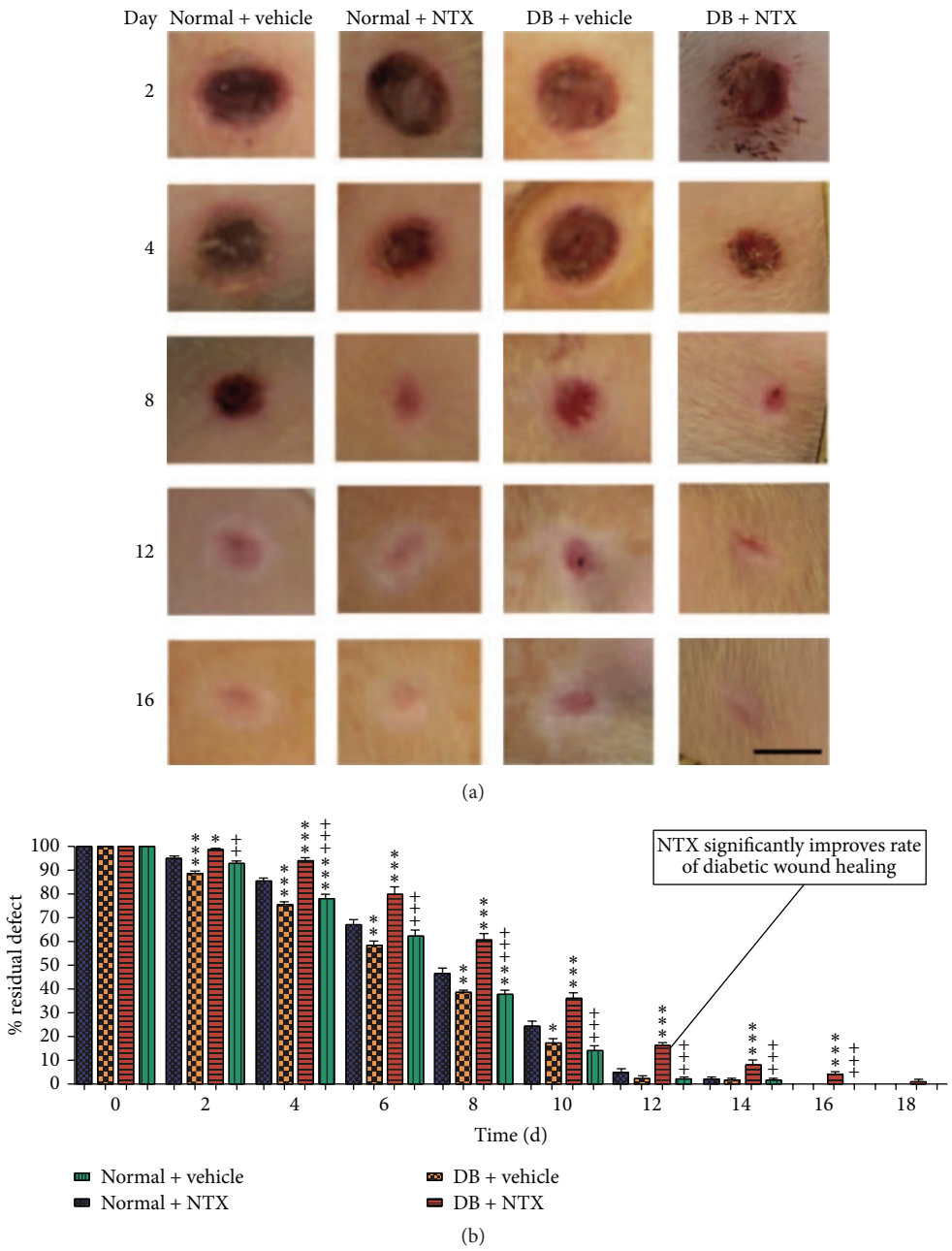

FIGURE 19: Full-thickness wound closure following application of topical NTX to normal and hyperglycemic rats. (a) Full-thickness wounds created on the dorsal surface of normal and type 1 diabetic rats. Wounds were treated three times daily with either 10^{-5} mol/L NTX (NTX) or saline (vehicle) dissolved in Neutrogena moisturizing cream. Photographs were taken every other day for 18 days. Bar = 5 mm. (b) Histograms of residual defects as a % of the original wounds. Groups were treated with either NTX or vehicle over an 18-day period of time. Diabetic NTX-treated rats (DB + NTX) had wounds 62–89% smaller compared to DB controls (DB + vehicle). Diabetic NTX-treated rats (DB NTX) had wounds 62–89% smaller than DB controls. Significantly different from normal + vehicle measurements at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; significantly different from DB + vehicle group at $^{++}P < 0.01$ and $^{+++}P < 0.001$. NTX: naltrexone; DB: diabetic (derived from [65]).

It must be noted that NTX treatment of standardized cutaneous wounds in the spontaneously diabetic db/db mouse model of type 2 diabetes also results in an increased labeling index and more rapid wound closure comparable to normal levels (Figure 21) [67]. It is interesting that, in these db/db mice, epithelium was hyperplastic in the skin of unwounded NTX-treated normal and DB rats compared to their counterparts (Figure 22) [67].

In summary, Opioid Growth Regulatory System blockade by NTX significantly and positively impacts cutaneous

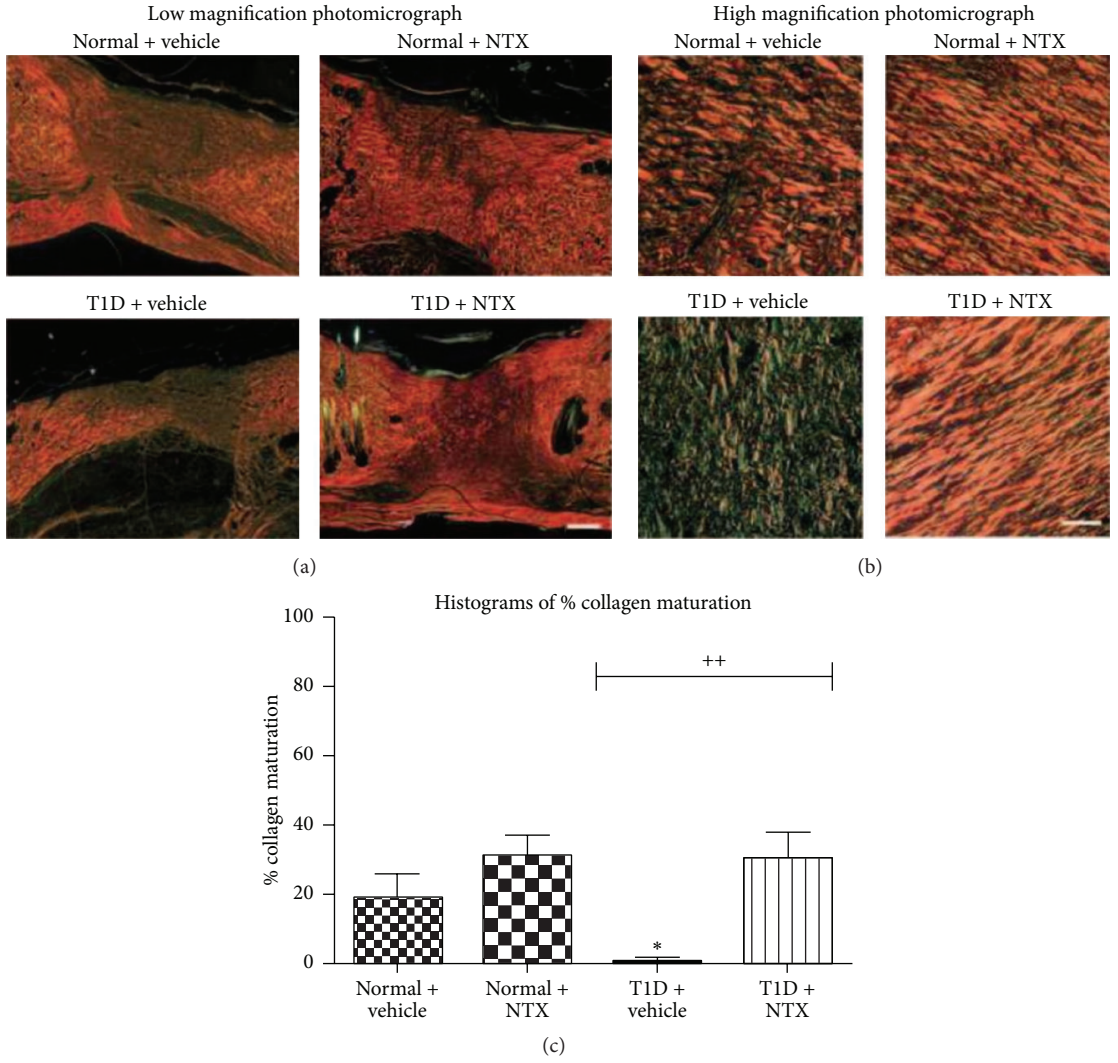

FIGURE 20: Sirius red-stained healing skin. Diminished collagen formation and maturation in diabetes are restored to normal by NTX treatment. These figures demonstrate remodeling of full-thickness wounds created in T1D and normal rats as measured by collagen formation in the reticular dermis 20 days after wounding and treatment with NTX dissolved in moisturizing cream or cream alone (vehicle). Wounds from normal and T1D rats were treated three times daily with either 10^{-5} M NTX or sterile saline (vehicle) dissolved in moisturizing cream. (a) Low magnification (4x) photomicrographs of Sirius red birefringence of skin sections encompassing the full-thickness wound and peripheral unwounded skin; bar = 100 mm. (b) High magnification of collagen maturation in Sirius red-stained sections described in (a); bar = 25 mm. (c) Histograms of the percent collagen maturation analyzed by ImageJ at 20 days. Values represent means ± SEM. *Significantly different from normal + vehicle values at $P < 0.05$; $^{++}$significantly different between T1D + vehicle and T1D + NTX at $P < 0.01$ (derived from [66]).

wound healing in diabetic animals thereby demonstrating its involvement in the pathobiology of systemic, nonocular diabetic complications.

5. Summary of Research and Implications

The Opioid Growth Regulatory System is a phylogenetically ancient growth regulatory system that has been conserved across multiple existing animal phyla and, specifically, in ocular tissue. It regulates cell division in all cell types capable of dividing that have been tested to date including normal, healing, embryologic, and neoplastic tissues.

The function of the Opioid Growth Regulatory System appears to be disordered in diabetic animals, and its function can be restored with NTX treatment to normalize corneal epithelial wound healing, corneal sensitivity, and tear production in models of both type 1 and type 2 diabetes. Moreover, studies by our team relative to cutaneous wound healing in diabetic animals further support the hypothesis that the Opioid Growth Regulatory System is disordered relative to wound closure, wound maturation, and the restoration of tissue tensile strength in nonocular tissue, specifically the skin. Thus, our findings support the hypothesis that the function of the Opioid Growth Regulatory System is diffusely and abnormally impacted by diabetes.

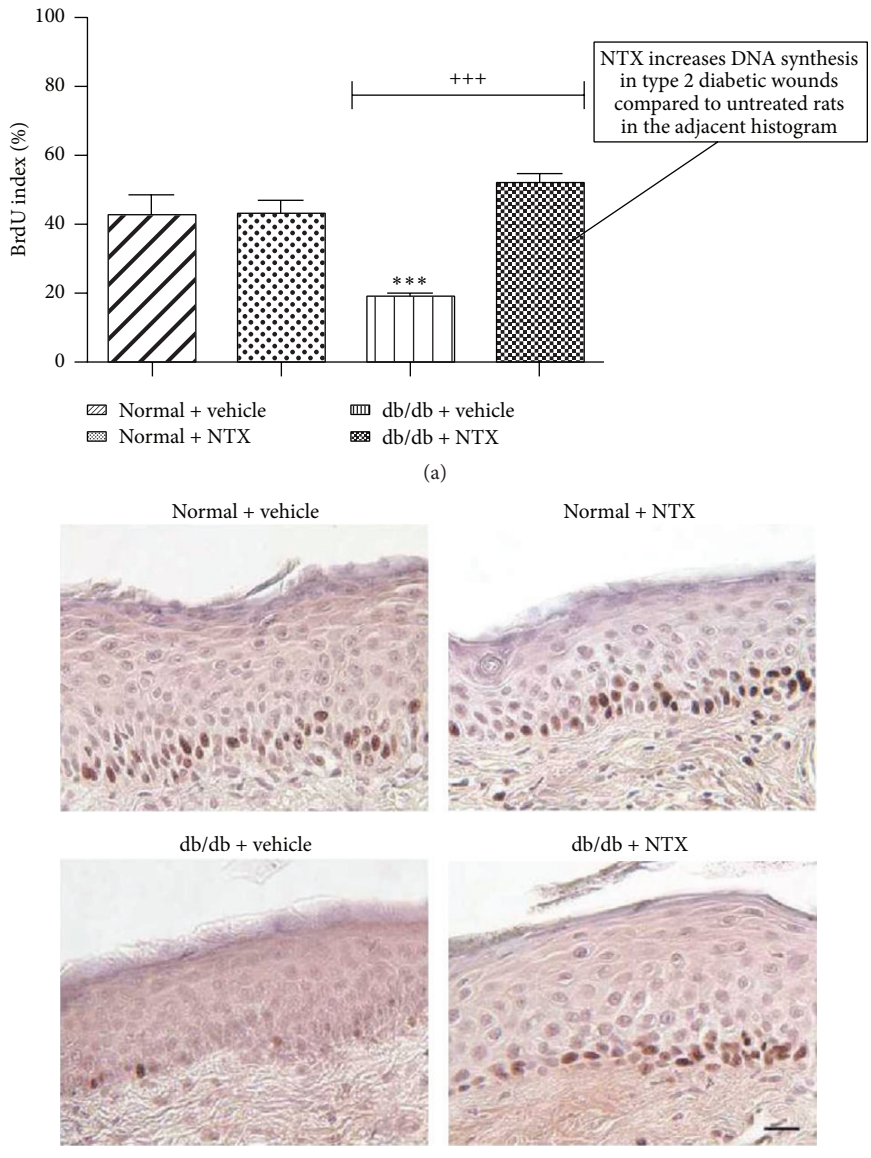

Figure 21: Spontaneously diabetic db/db mice with 5 mm full-thickness skin wounds on dorsum were treated with topical 10^{-5} M NTX in moisturizing cream or saline dissolved in moisturizing cream TID × 14 days. (a) DNA labeling indices calculated from the number of BrdU-positive basal cells relative to the total number of basal cells. Depressed DNA synthesis is noted in the DB rats compared to normal or NTX-treated DB animals. NTX-treated DB mice had BrdU LI of 52% compared to 43% for normal and NTX-treated normal animals. Values represent mean ± SEM. Significantly different from normal + vehicle group at $^{***}P < 0.001$. Labeling indexes in NTX-treated diabetic (db/db + NTX) skin were significantly different from measurements in saline-treated tissue from T2D mice (db/db + vehicle) at $^{+++}P < 0.001$. (b) Sections of the skin treated as above illustrate the relative labeling amounts of the treatment groups (derived from [67]).

Where do we go from here?

Naltrexone has been shown to be well tolerated in short-term ocular application in healthy human volunteers [68]. Further studies leading to the topical use of NTX in wound healing are required. Moreover, in our opinion, a more global study, to determine the impact of NTX therapy on the prevention of systemic complications of diabetes, is indicated.

Disclosure

This review was presented, in part, at the Lorenz. E Zimmerman Tribute Symposium, jointly sponsored by the American Academy of Ophthalmology and the American Association of Ophthalmic Oncologists and Pathologists, Chicago, Illinois, October 19, 2014. The paper was delivered by one of the authors, Joseph W. Sassani, M.D., but is

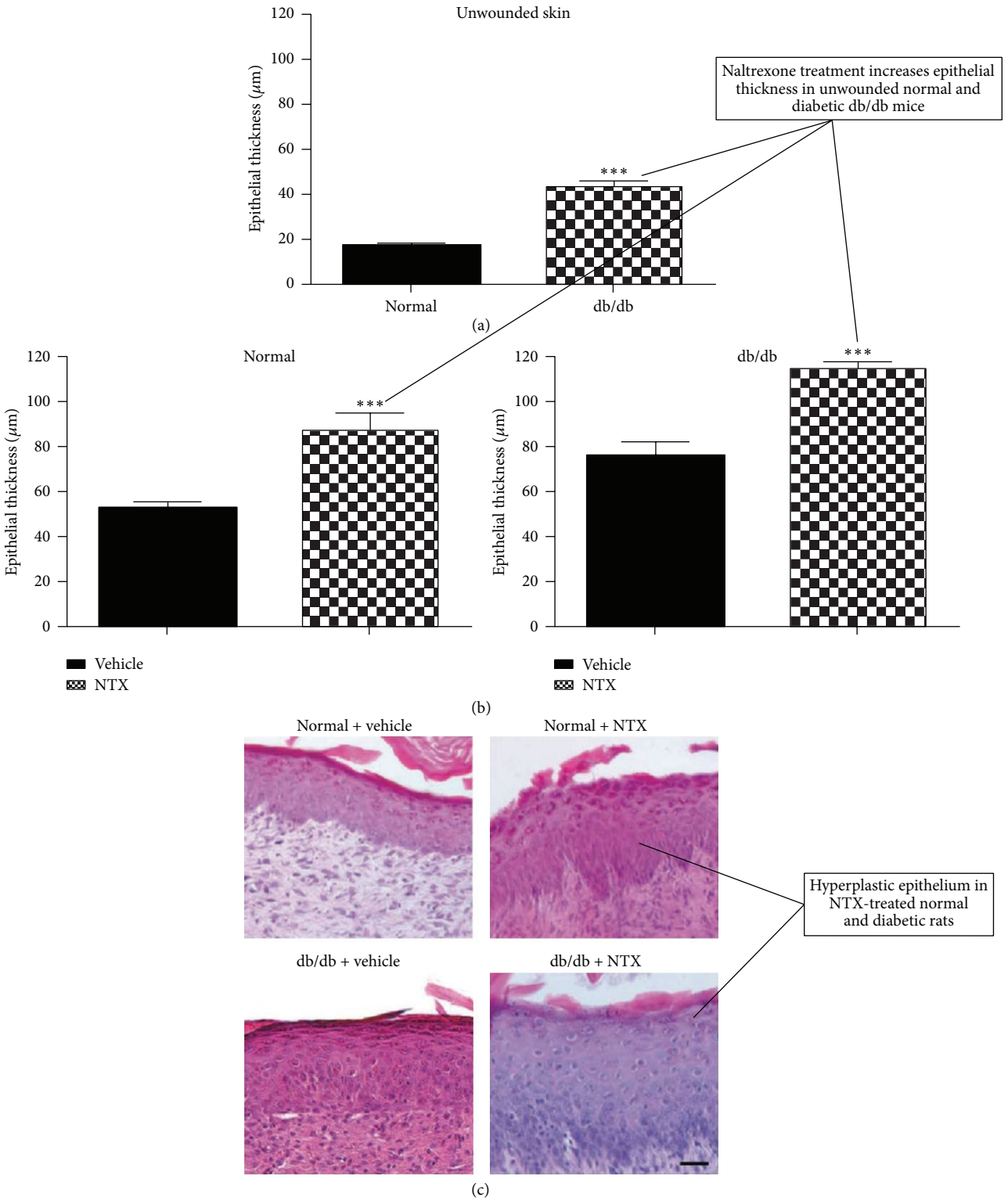

Figure 22: Histograms and photomicrographs illustrate the impact of 14 days of treatment with topical application three times daily of NTX or saline dissolved in Neutrogena moisturizing cream on the epithelialization of cutaneous wounds in normal and diabetic (db/db) mice. (a) The epithelium is hyperplastic in the NTX-treated normal and DB mice as illustrated in the histogram of epithelial thickness in normal and db/db unwounded skin. Values represent means ± SEM. Significantly different from normal values at $^{***}P < 0.001$. (b) Histograms of epithelial thickness over the underlying connective tissue in the wound bed in normal and db/db wounded skin treated with NTX or saline dissolved in moisturizing cream (vehicle). Values represent means ± SEM. Data from NTX-treated wounds were compared with vehicle-treated wounds for normal or db/db mice. Significantly different from vehicle-treated specimens at $^{***}P < 0.001$. (c) Photomicrographs of epithelium stained with hematoxylin and eosin permit comparison of the epithelial thickness among normal, diabetic NTX-treated, and control epithelium; bar = 25 μm (derived from [67]).

the result of research performed through the collaboration of all of the authors. Animal rights: all experiments were conducted in accordance with the National Institutes of Health guidelines on animal care and were approved by the Penn State Hershey Institutional Animal Care and Use Committee.

Competing Interests

The authors have patent interests through Penn State University in the clinical applications of the Opioid Growth Regulatory System.

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Review Article

Molecular and Electrophysiological Mechanisms Underlying Cardiac Arrhythmogenesis in Diabetes Mellitus

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Diabetes is a common endocrine disorder with an ever increasing prevalence globally, placing significant burdens on our healthcare systems. It is associated with significant cardiovascular morbidities. One of the mechanisms by which it causes death is increasing the risk of cardiac arrhythmias. The aim of this article is to review the cardiac (ion channel abnormalities, electrophysiological and structural remodelling) and extracardiac factors (neural pathway remodelling) responsible for cardiac arrhythmogenesis in diabetes. It is concluded by an outline of molecular targets for future antiarrhythmic therapy for the diabetic population.

1. Introduction

Cardiometabolic disorders place significant burdens on the healthcare system worldwide [1]. Their prevalence has been rising over the past decades due to an aging population and an increasing level of obesity [2, 3]. Diabetes mellitus is an endocrine disorder characterized by reduced insulin production (type 1) or increased insulin resistance (type 2), leading to hyperglycaemia. There is increasing evidence that diabetes increases the risk of cardiac arrhythmias. This involves abnormalities in action potential conduction or repolarization (Figures 1 and 2), due to a complex interplay of ion channel abnormalities and electrophysiological remodelling superimposed upon a cardiomyopathic process together with autonomic dysregulation (Figure 3). Some of these findings are derived from experiments performed in animal models, which have been proven extremely useful for dissecting the molecular mechanisms responsible for arrhythmic phenotypes [4]. In this review, the pathophysiology underlying cardiac arrhythmias in diabetes mellitus is explored in detail, followed by an outline of potential therapeutic targets for reducing arrhythmic risk and sudden death in diabetic patients.

2. Arrhythmogenic Mechanisms in Diabetes Mellitus

The common arrhythmogenic mechanism is reentry, which occurs when an action potential fails to extinguish itself and reactivates a region that has recovered from refractoriness. This can arise from abnormalities in conduction or repolarization or both [5]. Circus reentry requires three prerequisites: (i) conduction velocity (CV) which must be sufficiently slowed so that the tissue ahead of the action potential (AP) wavefront remains excitable, (ii) unidirectional conduction block which must be present to prevent waves from self-extinguishing when they collide, and (iii) an obstacle around which an AP can circulate [6]. This need not be a structural defect but can be a functional core of refractory tissue, which may arise dynamically from ectopic activity [7]. Repolarization abnormalities can result in early or delayed afterdepolarizations (EADs and DADs), which can initiate triggered activity when their magnitudes are sufficiently large to reach the threshold potential for sodium channel reactivation. They can also increase the dispersion of repolarization, promoting unidirectional conduction block and reentry. In diabetes mellitus, arrhythmogenesis can be due to

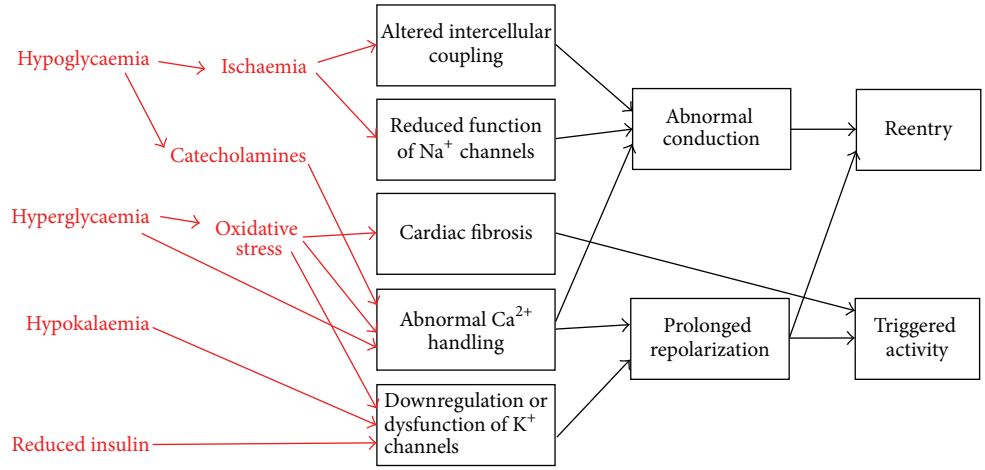

Figure 1: Both conduction and repolarization abnormalities promote arrhythmogenesis in diabetes.

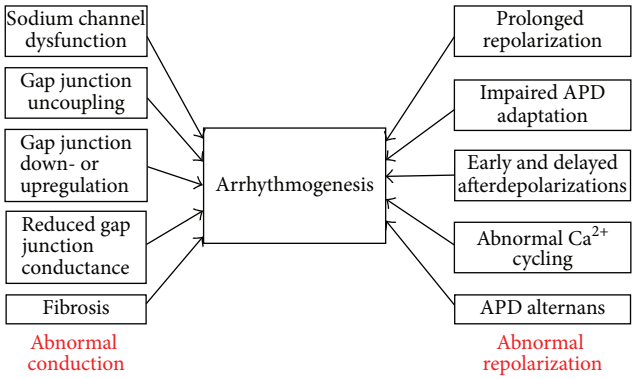

Figure 2: Cardiac and extracardiac factors responsible for promoting arrhythmogenesis in diabetes.

the following mechanisms. Abnormalities in conduction are mediated by myocardial ischaemia [8] or in repolarization [9, 10] by ion channel dysfunction, increased adrenergic drive, and calcium overload [11]. These abnormalities are superimposed upon a cardiomyopathy, in which the structural changes also predispose to arrhythmias. Extracardiac abnormalities, for example, neural pathway remodelling, can further promote arrhythmogenesis [12]. Ventricular arrhythmias are thought to underlie sudden cardiac death (SCD) in type 2 diabetic patients and also the "dead-in-bed syndrome" observed in otherwise young healthy adults with type 1 diabetes [13].

3. Abnormal Conduction

CV depends upon sodium channel activation followed by electrotonic spread of the ionic currents via gap junctions, which are electrical coupling pathways located between adjacent cardiomyocytes [14]. Each gap junction is made of two connexons, and each connexon is a hexamer of connexins (Cx). Altered gap junction expression or function can produce conduction abnormalities and in turn predispose to reentrant excitation. Protein kinase C- (PKC-) mediated phosphorylation, a calcium-dependent process, at serine 368 of Cx43, has been linked to reduced gap junction conductance [15, 16]. Dephosphorylation of gap junctions results in their uncoupling [17] and lateralization [18, 19]. There is consistent evidence demonstrating altered gap junction function or expression in different experimental models of diabetes. Thus, in transgenic mice with cardiac-specific overexpression of peroxisome proliferator-activated receptor γ 1 (PPARγ1) modelling human diabetes, reduced Cx43 expression without alterations in CV was observed [20]. This may increase anisotropy and higher likelihood of reentry. In streptozotocin- (STZ-) induced diabetic rats, expression levels of Cx40, 43 and 45 in the SA node, are significantly increased, which were associated with SA conduction delay [21]. This can be explained by increased expression levels of Cx45, which has the lowest unitary conductance and whose expression reduces CV. In both atria and ventricles of the same model, Cx43 phosphorylation was decreased because of reduced PKCε expression [22]; Cx43 was upregulated in the atria, whereas its expression level was unchanged in the ventricles [23]. Furthermore, the lack of insulin signalling can lead to reduced CV of propagating APs.

Myocardial fibrosis is increasingly recognized to be a pathogenic factor in diabetic cardiomyopathy [24]. Fibrosis resulting from fibroblast activation is mediated by growth factors, such as transforming growth factor-β [25]. This produces conduction abnormalities via two mechanisms: (i) reduced coupling between cardiomyocytes, leading to increased axial resistance; (ii) increased coupling between fibroblast and cardiomyocyte, increasing membrane capacitance [26]. Both mechanisms lead to a decrease in CV. Cardiac magnetic resonance (CMR) with late gadolinium enhancement is used for the diagnosis and monitoring of cardiomyopathy [27–29] and is potentially useful for examining fibrosis in diabetic cardiomyopathy.

Hypoglycemic episodes are associated with myocardial ischaemia [8], which may predispose to ventricular

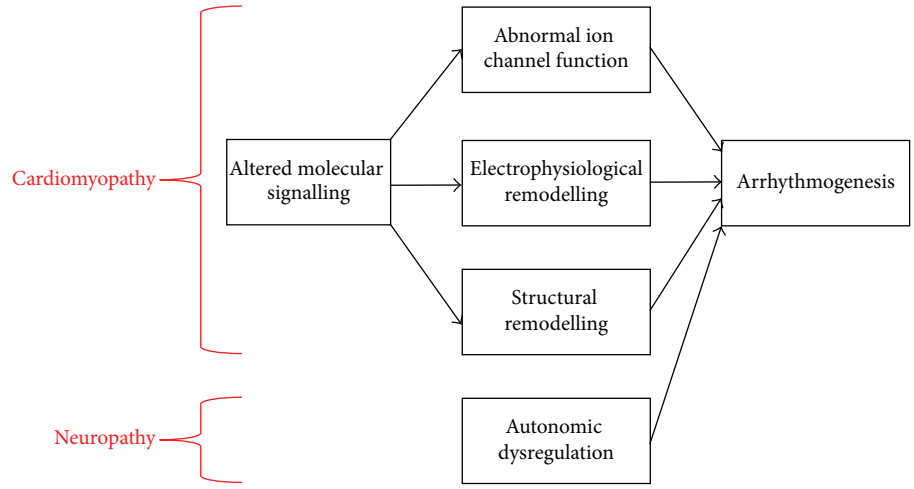

Figure 3

arrhythmias by producing conduction defects via the following mechanisms [14]. Ischaemia results in ATP depletion, metabolic switching to anaerobic glycolysis, extracellular H^+ accumulation, and intracellular Ca^{2+} overload. Cytosolic Ca^{2+} binds to the conserved C2 domain of PKC, thereby activating it [30]. There are several downstream targets of PKC. Firstly, PKC phosphorylates the serine residue at 1505 position of the sodium channel inactivation gate between domains III and IV, which decreases I_{Na} [31]. Secondly, it also phosphorylates connexins (Cx) 43 at serine 368, reducing gap junction conductance [15, 16]. Ca^{2+} overload is also associated with dephosphorylation of gap junctions [32], resulting in their uncoupling [17] and lateralization [18, 19]. Thus, myocardial ischaemia secondary to hypoglycaemia reduces CV and increases dispersion of conduction, predisposing to reentrant excitation.

4. Abnormal Repolarization

Action potential repolarization has two phases: (i) early rapid repolarization resulting from the activation of the fast and slow transient outward potassium currents, $I_{to,f}$ and $I_{to,s}$, and (ii) prolonged plateau resulting from a balance between the inward currents mediated by the voltage-gated L-type calcium channel (LTCC, $I_{Ca,L}$) and sodium-calcium exchanger (I_{NCX}) and the outward currents mediated by the voltage-gated delayed rectifier potassium channels (I_K: rapid and slow currents, I_{Kr} and I_{Ks}) [33]. There is also contribution from the inward rectifying current (I_{K1}). Of these, the human ether-à-go-go-related gene (HERG) K^+ channel is the major component of delayed rectifier K^+ current [34].

In diabetes mellitus, prolongations in action potential durations (APDs) are due to several mechanisms. The lack of insulin signalling resulted in electrophysiological remodelling: I_{to} is reduced as a result of reduced expression of Kv4.2 and KChiP2 genes [35]. This current is posttranslationally regulated by a number of different kinases. For example, the p90 ribosomal S6 kinase (p90RSK) is a serine/threonine kinase with N- and C-terminal kinase domains. Reactive oxygen species (ROS), which are raised in diabetes [36], increases the activity of p90RSK and reduced the activity of $I_{to,f}$, $I_{K,slow}$, and I_{SS} channels [37]. Moreover, transgenic mice with cardiac-specific overexpression of peroxisome proliferator-activated receptor γ 1 (PPARγ1) showed abnormal lipid accumulation in cardiomyocytes and reduced expression as well as function of $I_{to,f}$ and $I_{K,slow}$[20]. The Rad (Ras associated with diabetes) protein is implicated in diabetes: in its dominant negative mutant, LTCC was upregulated [38]. Together, increased inward currents and decreased outward currents lead to prolonged ventricular repolarization. Conversely, genetic mutations of key ion channel genes causing prolonged ventricular repolarization can also lead to diabetes. For example, mutations in KCNE2 are responsible for long QT syndrome type 5. Whole-transcript transcriptomics demonstrated that $KCNE2^{-/-}$ mice additionally showed diabetes mellitus, hypercholesterolemia, and elevated angiotensin II levels [39]. Hypoglycaemia causes intracellular depletion of ATP in cardiomyocytes and hyperglycaemia increases the production of reactive oxygen species (ROS), both leading to HERG channel dysfunction [40]. K_{ATP} channels are thought to provide a link between cellular energy status and membrane electrophysiology. They are normally inhibited by ATP and activated by ADP. During ischaemia, there are ATP depletion and ADP accumulation, activating $I_{K,ATP}$ and promoting APD shortening [41]. In diabetes, initial APD shortening is also observed but this becomes fully reversed in a time-dependent manner. This failure of APD adaptation, when accompanied by increased adrenergic drive, can engage in steep APD restitution, in turn leading to the production of arrhythmogenic APD alternans [7].

Hypoglycaemia is also associated with another cause of delayed repolarization, hypokalaemia [42, 43], which arises from insulin therapy or increased adrenergic drive [44, 45]. Hypokalaemia inhibits I_{K1}, thereby prolonging APDs and causing L-type Ca^{2+} channel reactivation [46]. This then leads to early afterdepolarizations (EADs) and consequent triggered activity [47]. Hypokalaemia also preferentially prolongs epicardial APDs and leaving endocardial APDs

unchanged, increasing the transmural repolarization gradient [47]. In combination with reduced effective refractory periods (ERPs), excitation wavelength (conduction velocity (CV) × ERP) is reduced. Furthermore, increased steepness of APD restitution results in the development of APD alternans [48] and in turn in wavebreak, conduction block, and initiation and maintenance of reentrant activity [7, 49].

Hypoglycaemia also increases adrenergic drive with the following proarrhythmic consequences [50]. Firstly, the release of catecholamines leads to abnormal Ca^{2+} cycling and intracellular Ca^{2+} accumulation. This in turn stimulates spontaneous Ca^{2+} release from the sarcoplasmic reticulum, thereby activating three calcium-sensitive currents: the nonselective cationic current, I_{NS}, the sodium-calcium exchange current, I_{NCX}, and the calcium-activated chloride current, $I_{Cl,Ca}$. Thus, such inward currents observed during phase 4 of the action potential lead to delayed afterdepolarizations (DADs), eliciting triggered activity.

Abnormal Ca^{2+} dynamics have been implicated in diabetes. For example, cardiomyocytes of leptin-deficient ob/ob mice showed reduced amplitudes of Ca^{2+} transients, and insulin elicited extra transients via inositol 1,4,5-trisphosphate (IP_3) signalling and impaired mitochondrial Ca^{2+} handling [51]. Furthermore, decreases in DAG-mediated nonselective cation currents were associated with reduced TRPC3 expression at the plasma membrane, which increases Ca^{2+} influx [52]. Dysregulation of the type 2 ryanodine receptor (RyR2) has been detected in a STZ-induced diabetes rat model, in which increased frequency of Ca^{2+} sparks with reduced amplitudes was associated with increased sensitivity to Ca^{2+} activation and dyssynchronous Ca^{2+} release [53, 54]. Abnormal RyR2 gating mechanism may arise from increased phosphorylation by protein kinase A (PKA, serine 2808) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII, serine 2808 and serine 2814) [55–57], as well as oxidation by ROS and reactive carbonyl species (RCS), which are increased in diabetes [58–60]. Uncontrolled hyperglycaemia can lead to activation of CaMKII and subsequent Ca^{2+} release from the SR [61]. Dyssynchronous Ca^{2+} release can be explained by remodelling of the transverse tubular system, whereby RyR2 become orphaned when they are decoupled from LTCCs [62]. Interestingly, catecholaminergic polymorphic ventricular tachycardia (CPVT) is caused by RyR2 mutation, and patients suffering from this condition are also prone to impaired glucose homeostasis and insulin secretion [63]. It would be interesting to determine whether diabetic patients with acquired dysfunction in RyR2 develop bidirectional VT classically associated with CPVT.

Moreover, diabetes mellitus is an independent risk factor for atrial fibrillation, yet the underlying physiological mechanisms are incompletely understood. It may involve ion channel remodelling in the atria. For example, the small conductance Ca^{2+}-activated K^+ (SK) channels contribute to atrial repolarization. SK2 and SK3 isoforms are downregulated, leading to APD prolongation [64]. Normally, SK channels do not play a role in ventricular repolarization. In heart failure, SK currents and ion channel expression can be upregulated and become more sensitive to Ca^{2+} modulation, potentially leading to ventricular arrhythmias [65]. Altered expression of SK channels in the ventricles may play a role in diabetes but this remains to be tested experimentally.

5. Diabetic Cardiomyopathy: Cardiac Electrophysiological and Structural Remodelling with Superimposed Autonomic Dysregulation

Diabetic cardiomyopathy is characterized by diastolic dysfunction with preserved systolic function, findings that are similarly observed in genetically modified, leptin receptor deficient, diabetic db/db mice on echocardiography [66, 67]. Cardiac magnetic resonance imaging is excellent for characterizing structural abnormalities, such as areas of fibrosis by late gadolinium enhancement [27–29]. Afferent and efferent neural pathways normally regulate inotropic, lusitropic, chronotropic, and dromotropic responses of the heart. In diabetes, these can become dysregulated with impaired baroreceptor control of heart rate [68]. Reduced heart rate variability (HRV) has long been associated with increased mortality [69]. In diabetes, a reduction in HRV was associated with increased incidence of inducible VT by programmed electrical stimulation [70]. Electrophysiological modelling is likely to be an early event, appearing before structural abnormalities. Thus, STZ-induced diabetic rats showed decreases in both maximal transport capacity of SERCA2a and RyR2 conductance, associated with impairment of both inotropic and lusitropic responses in response to adrenergic stimulation [71]. This finding differs from human findings with impaired positive inotropic response with preservation of positive lusitropic effects of beta-adrenoceptor stimulation [72].

Brady-arrhythmias in the form of sinoatrial (SA) and atrioventricular (AV) nodal blocks are seen in diabetes [73, 74]. Sinoatrial node (SAN) dysfunction was demonstrated in db/db mice, which demonstrated prolonged SAN recovery time [66]. These mice showed no significant differences in conduction intervals and wave amplitudes compared to control mice. By contrast, sinus tachycardia at rest has been associated with excessive mortality in diabetic patients [75]. This may be related to autonomic dysregulation, with increased adrenergic drive with or without impairment of parasympathetic response. Thus, in Akita diabetic mice, the SA node is less responsive to acetylcholine because of a reduction in acetylcholine-activated K^+ current ($I_{K,ACh}$), which is due to altered phosphoinositide 3-kinase (PI3K) signalling [76].

Some aspects of altered cardiac electrophysiology in diabetes do not arise from abnormalities in the heart itself, but instead from neural pathways innervating it. Thus, in STZ-induced diabetic mice, both baroreflex tachycardia and bradycardia were blunted. This was associated with remodelling of the baroreceptor circuitry, in which the sizes of cardiac ganglia and ganglionic principal neurons were decreased. In a different model, the OVE26 diabetic mice showed neural degeneration in the nucleus ambiguus, which is one of the two brainstem nuclei innervating the cardiac

Table 1

Molecular target	Mechanism of action	References
Gap junction inhibitors	Increase refractory period Improve conduction	[47]
Gap junction openers	Increase conduction velocity and decrease heterogeneity in repolarization or refractoriness	[49]
Late sodium channel blockers	Inhibit afterdepolarizations	[83]
Ryanodine receptor stabilizers	Decrease heterogeneity in Ca^{2+} transients and inhibit afterdepolarizations	[84]
Antifibrotic agents	Reduce cardiac fibrosis	[82]

ganglia [77]. Furthermore, altered balance between chemoattractants (e.g., nerve growth factor) and chemorepellants (Sema3a) leads to disruptions in innervation pattern, precipitating arrhythmias, and sudden death [78].

6. Clinical Relevance and Future Therapies

Traditional agents used for treatment of diabetes or associated comorbidities such as hypertension have been shown to exert cardiac protective effects in diabetes by previously unknown mechanisms. Thus, for example, in the STZ-induced diabetic rat model, I_{to} and I_{SS} are downregulated and the cardiac renin-angiotensin system is activated. Experimental evidence has demonstrated augmentation of both currents by the antihypertensive angiotensin II receptor blockers [79]. The ACE inhibitor enalapril [80] and angiotensin II receptor blocker losartan [81] were also shown to exert antifibrotic effects in hypertension and may have similar cardioprotective effects in diabetes by similar mechanisms. The antifibrotic hormone relaxin could be delivered using adenoviruses [82] and may reverse fibrosis in diabetic cardiomyopathy. Ion channels represent an attractive target for managing arrhythmic complications of diabetes mellitus (Table 1). Novel agents such as late sodium current blockers [83] and gap junction openers [49] can be used to reduce abnormal repolarization and conduction, respectively. Alternatively, gap junction inhibitors can prolong effective refractory periods and exert antiarrhythmic effects [47]. Paradoxically, mild gap junction uncoupling could improve the safety margin of conduction and increase CV, removing unidirectional conduction blocks and converting these into bilateral conduction. Their use in diabetes warrants future exploration. Ryanodine receptor stabilizers have the potential to normalize Ca^{2+} handling in diabetes, which remains to be tested [84]. However, caution must be exercised to screen for deleterious, ventricular proarrhythmic effects. K_{ATP} channels play a role in not only insulin secretion but also cardiac repolarization. Whilst the K_{ATP} channel activators have been used to increase insulin release, they have the potential to cause life-threatening ventricular arrhythmias, especially in a subset of patients with ischaemic complications. In diabetes, mitochondrial K_{ATP} channel activation in cardiomyocytes by dioxide led to impaired APD adaptation, which promoted the occurrence of VT [85]. Future efforts therefore require an integrated approach by computation modelling, where effects of drugs on complex spatiotemporal properties of cardiac dynamics are tested to reduce the likelihood of life-threatening side effects. Animal models will be useful for studying arrhythmogenic mechanisms and provide a platform for assessing the efficacy of pharmacological therapy with translational applications [86–88].

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Coronary Plaque Characteristics Assessed by 256-Slice Coronary CT Angiography and Association with High-Sensitivity C-Reactive Protein in Symptomatic Patients with Type 2 Diabetes

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Little is known regarding plaque distribution, composition, and the association with inflammation in type 2 diabetes mellitus (DM2). This study aimed to assess the relationship between coronary plaque subtypes and high-sensitivity C-reactive protein levels. Coronary CTA were performed in 98 symptomatic DM2 patients and 107 non-DM2 patients using a 256-slice CT. The extent and types of plaque as well as luminal narrowing were evaluated. Patients with DM2 were more likely to have significant stenosis (>50%) with calcified plaques in at least one coronary segment ($p < 0.01$); the prevalence rates of diffuse calcified plaques in the DM2 and non-DM2 groups were 31.6% and 4.7%, respectively ($p < 0.01$). Plasma hs-CRP levels in DM2 with calcified plaques were higher compared with values obtained for the non-DM2 group ($p < 0.01$). In conclusion, combination of coronary CTA and hs-CRP might improve risk stratification in symptomatic DM2 patients.

1. Introduction

Diabetes mellitus is the most important risk factor for coronary artery disease (CAD). Type 2 diabetes mellitus (DM2) has reached epidemic proportions globally, affecting populations of both developed and developing countries [1]. CAD is often asymptomatic in DM2 patients until the onset of myocardial infarction or sudden cardiac death [2]. It has been widely demonstrated that acute cardiac events are related to rupture and acute thrombosis caused by a mildly stenotic plaque, namely, the vulnerable plaque [3]. Major advances in CAD prevention require early detection of the vulnerable plaques.

Conventional X-ray coronary angiography is the current gold standard for invasive evaluation of CAD. However, it only shows the lumen of the vessel, greatly underestimating the atherosclerosis burden. A noninvasive assay to directly detect coronary atherosclerosis would therefore be beneficial. Coronary CTA provides comprehensive information noninvasively regarding the location, severity, and characteristics of coronary atherosclerotic plaques: noncalcified, calcified, and mixed plaques can be identified. A previous study showed that vulnerable plaques in diabetic patients tend to occur at multiple sites, with high atherosclerotic burden [4].

Inflammation is a fundamental component of atherosclerosis [5, 6]. The most widely tested inflammatory biomarker is high-sensitivity C-reactive protein (hs-CRP), which predicts the risk of a first MI in healthy individuals and future coronary events in patients with stable CAD [7]. It is well known that both elevated CRP and specific plaque subtypes are associated with poor disease outcome [8, 9]. Understanding how CRP and vulnerable plaques are related and using imaging

techniques to assess this relationship may enable the early identification of vulnerable patients. Recently, in a cohort of asymptomatic subjects, increased CRP levels were found to be associated with the prevalence of any plaque and mixed calcified plaques, as well as significant coronary stenosis [10]. To the best of our knowledge, no study has been reported regarding symptomatic diabetic patients. Therefore, we aimed to assess the relationship between hs-CRP levels and plaque subtypes in symptomatic patients with DM2 using 256-slice CT.

2. Methods

2.1. Study Population and Design. From December 2013 to December 2014, 205 patients underwent 256-slice CT coronary angiography, including 98 DM2 cases and 107 individuals without DM2. They were 108 men and 97 women, 48 ± 15 years old, with BMI of 22.15 ± 2.36 kg/m^2. No patient had a known previous CAD. Serum hs-CRP levels were measured before coronary CTA. The diagnostic criteria for DM2 were based on WHO guidelines.

Exclusion criteria were heart rate ≥ 90 bpm, atrial fibrillation, severe renal insufficiency (serum creatinine > 120 μmol/L), severe respiratory insufficiency, hyperthyroidism, and allergy to iodine-based contrast. The study was approved by the Institutional Review Board of the hospital, and informed consent was obtained from each patient.

2.2. Instruments, Equipment, and Scanning Method. CTA examinations were performed on a 256-slice CT (Brilliance iCT; Philips, Amsterdam, Netherlands) and a power injector (SCT-211; Medrad Inc., Indianola, PA, USA). The scan protocols were as follows: detector width, 80 mm; detector collimation, 128 × 0.625 mm; slice acquisition, 128 × 0.625 mm using a z-flying focal spot; gantry rotation time, 0.27 s. Tube voltage and current were set at 300–500 mAs per rotation and 120–140 kVp, respectively. The contrast material (Ultravist Solution 370 mg I/mL; Bayer Healthcare, Berlin, Germany) was intravenously injected through an antecubital vein using a 20-gauge needle connected to a power injector (SCT-211; Medrad Inc., Indianola, PA, USA). Contrast material injection timing was controlled by the bolus-tracking technique in the ascending aorta (signal attenuation threshold 100–120 Hounsfield units [HU]). Data acquisition was initiated with a mean delay of 6 s after reaching the threshold in the ascending aorta. A total amount of 60–80 mL of contrast material was injected at 5 mL/s followed by 30 mL of saline chaser. The mean effective radiation dose of the coronary CTA was 1.58 ± 0.36 mSv.

2.3. Coronary CTA Images Analysis. All scans were analyzed independently by two experienced radiologists blinded to the clinical information, on a Brilliance workstation (Philips Healthcare, Amsterdam, Netherlands). Each lesion was identified using the multiplanar reconstruction technique and free mode maximum intensity projection.

The 15 coronary segments were defined according to American Heart Association (AHA) standards. Lesions were classified by the maximal luminal stenosis observed on any plane; grading of stenosis was further classified as normal appearing (<25%) and mild (25%–49%) and moderate (50%–74%) and severe (≥75%) narrowing. Plaques occupied by calcified tissue >50% of the plaque area (density > 130 Hounsfield units in native scans) were classified as calcified arterial plaques (CAP); those with <50% calcium were considered mixed calcified arterial plaques (MCAP); and plaques without calcium were classified as noncalcified arterial plaques (NCAP) as previously described (Figure 1).

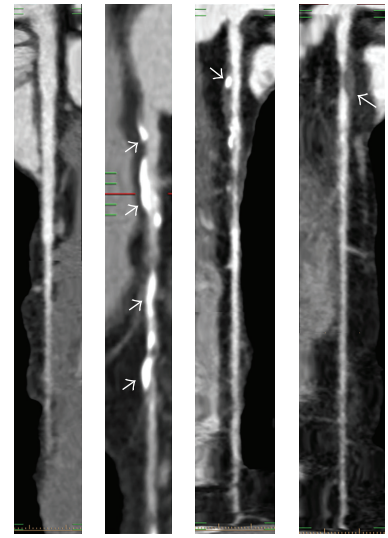

Figure 1: Representative CCTA images showing the assessed plaque subtypes. (a) Normal; (b) calcified arterial plaques; (c) mixed calcified arterial plaque; and (d) noncalcified arterial plaques.

2.4. Statistical Analysis. Continuous variables are mean ± standard deviation (SD) and categorical variables as number and percentage. The extent and types of plaque as well as luminal narrowing were evaluated and compared between diabetic and nondiabetic patients. Groups were compared using Student's t-test and Chi-square test. The patients were further divided into 3 groups according to median hs-CRP levels: low/normal group (hs-CRP ≤ 1 mg/L), intermediate group (1 mg/L < hs-CRP ≤ 2 mg/L), and high group (>2 mg/L). Multivariate logistical regression analysis was performed to explore the relationship between diabetes, CRP, and type and extent of plaque. Statistical analyses were performed with the SPSS 18.0 software (SPSS, Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Baseline Characteristics. The patients were 48 ± 15 years old and included 52.7% men; body mass index values were 22.15 ± 2.36 kg/m^2. The average DM2 duration was 8.5 ± 7.8 years. Compared with subjects without DM2, no significant differences in the levels of total cholesterol, triglyceride, high-density lipoprotein cholesterol, and low-density lipoprotein

Table 1: Basic characteristics.

Characteristic	All patients	DM2 patients	Non-DM patients
Number (n)	205	98	107
Age (years)	48 ± 15	46 ± 14	44 ± 13
Male (%)	108 (52.7)	55 (56.1)	48 (44.9)
BMI (kg/m^2)	22.15 ± 2.36	23.22 ± 1.37	22.43 ± 2.14
Mean heart rate	65 ± 13	64 ± 11	63 ± 12
Hypertension (%)	131 (63.9)	78 (79.6)	53 (49.5)
Current smoking (%)	82 (40.0)	40 (40.8)	42 (39.3)
Family history of coronary disease (%)	116 (56.6)	55 (56.1)	61 (57.0)
TC (mmol/L)	4.82 ± 1.12	4.85 ± 1.09	4.86 ± 1.14
TG (mmol/L)	2.18 ± 0.81	2.09 ± 1.02	1.91 ± 0.78
HDL-C (mmol/L)	1.24 ± 0.28	1.19 ± 0.23	1.34 ± 0.26
LDL-C (mmol/L)	2.96 ± 0.45	2.92 ± 0.63	2.94 ± 0.46

Data are mean ± SD or n (%). BMI: body mass index; TC: total cholesterol, TG: triglyceride, HDL-C: high-density lipoprotein cholesterol, and LDL-C: low-density lipoprotein cholesterol.

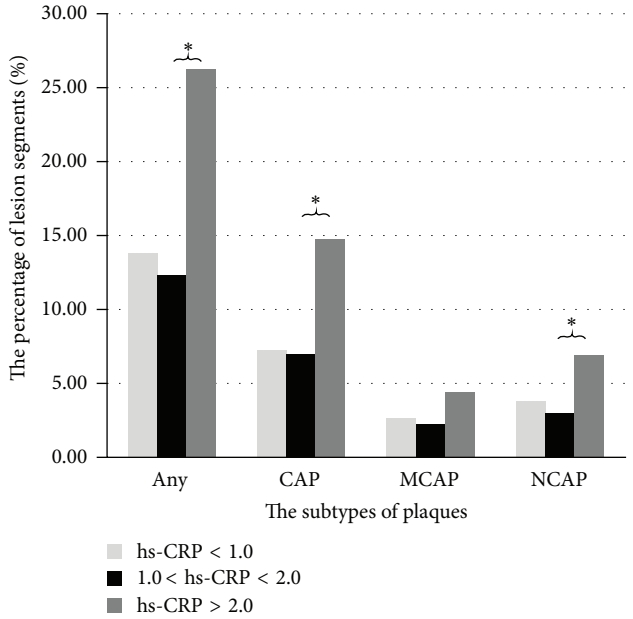

Figure 2: Percentage of patients with various plaque subtypes according to CRP category. Subjects with high hs-CRP levels were more likely to have any plaque, CAP, or NCAP compared with the second and third tertiles. ∗ refers to a statistically significant association between hs-CRP and the subtype of plaques.

cholesterol were obtained. Table 1 summarizes the general characteristics of the study population.

3.2. Coronary Artery Plaque Subtypes and Distribution. A total of 432 coronary vessels (4.3 ± 0.18 per patient) and 820 segments (8.2 ± 0.06 per patient) had plaques. In total, 1194 plaques (5.97 ± 0.22 per patient) were detected. Figure 1 depicts the three coronary plaque subtypes found in this study. Calcified plaques (54.7%) were more frequently detected than mixed or noncalcified ones ($p < 0.001$). The most commonly affected coronary vessel was the LAD artery in both groups: 40.4% LAD, 27.1% RCA, 18.3% LCX, and 14.2% LM were diseased in diabetics, with 39.0% LAD, 32.1% RCA, 18.2% LCX, and 10.7% LM in nondiabetic patients (all $p < 0.001$).

3.3. DM2 Patients versus Non-DM2 Patients. 262 diseased coronary vessels with 453 affected segments were found in diabetic patients, whereas 170 diseased coronary vessels and 367 affected segments were observed in nondiabetic individuals. Diffuse vessel disease was more commonly detected in DM2 patients than nondiabetic subjects (31.6% versus 4.7%; $p < 0.01$). In addition, more diseased segments were found in patients with diabetes (8.23 ± 4.48) compared with nondiabetic subjects (3.67 ± 2.42, $p < 0.05$). Furthermore, CAD tended to be more severe in DM2 patients as both left main/LAD coronary artery and multivessel diseases were more frequently diagnosed, although the difference was not statistically significant. More calcified plaques and less noncalcified plaques were detected in diabetics compared with nondiabetic patients, respectively (72.9% versus 48.1%, $p < 0.001$; 27.1% versus 51.9%, $p < 0.001$). As DM2 disease time increased from 5 to 10 and 10 to 15 years, the proportion of calcified plaques increased, with that of noncalcified plaques decreasing significantly (63.1% versus 24.0%, $p < 0.001$). Obstructive plaques were more abundant in diabetic patients compared with nondiabetics (28.3% versus 7.6%, $p < 0.05$). The plaque burden and stenosis data are shown in Table 2.

3.4. Prevalence of Coronary Plaques Based on CRP. Figure 2 depicts the prevalence of any coronary plaque subtype according to the various CRP cutoffs. Subjects with high CRP levels were more likely to have any plaque, CAP, or NCAP compared with individuals showing normal CRP levels ($p < 0.01$). In contrast, no significant difference was obtained for MCAP. Plasma CRP levels were significantly higher in individuals with DM2 compared with controls (3.232 ± 0.327 mg/L versus 1.937 ± 0.198 mg/L, $p < 0.01$). Figure 3 shows a symptomatic DM2 patient with increased CRP, in whom coronary CTA revealed triple vessel disease with diffuse calcified plaques.

3.5. Relationship between Diabetes, CRP, and Type and Extent of Plaque. Both the unadjusted and the multivariable logistic regression analyses for the presence of any coronary plaque and plaque subtype are listed in Table 3. Subjects were divided into groups according to CRP levels, and all analyses were performed using the low-normal CRP group as the reference category. Subjects with high CRP were observed to be at increased risk for the presence of CAP in the unadjusted

Table 2: Comparison of plaque burden and grading of stenosis between diabetic and nondiabetic patients.

	All patients	DM2	Non-DM	p value
Plaque burden	1194	826	368	
Calcified plaque**	653 (54.7%)	476 (57.6%)	177 (48.1%)	<0.01
Noncalcified plaque	330 (27.6%)	207 (25.1%)	123 (33.4%)	0.021
Mixed calcified plaque	211 (17.7%)	143 (17.3%)	68 (18.5%)	0.046
Grading of stenosis				
Normal appearing (<25%)	2180 (72.8%)	1047 (69.8%)	1133 (75.5%)	<0.01
Mild narrowing (25%–49%)	563 (18.7%)	287 (19.1%)	276 (18.4%)	0.042
Moderate narrowing (50%–74%)	181 (6.0%)	106 (7.1%)	75 (5.0%)	0.039
Severe narrowing** (≥75%)	76 (2.5%)	60 (4.0%)	16 (1.1%)	<0.01
Nonobstructive plaques	765 (64.1%)	485 (40.6%)	281 (23.5%)	0.028
Obstructive plaques*	429 (35.9%)	338 (28.3%)	91 (7.6%)	<0.05

* $p < 0.05$; ** $p < 0.01$.

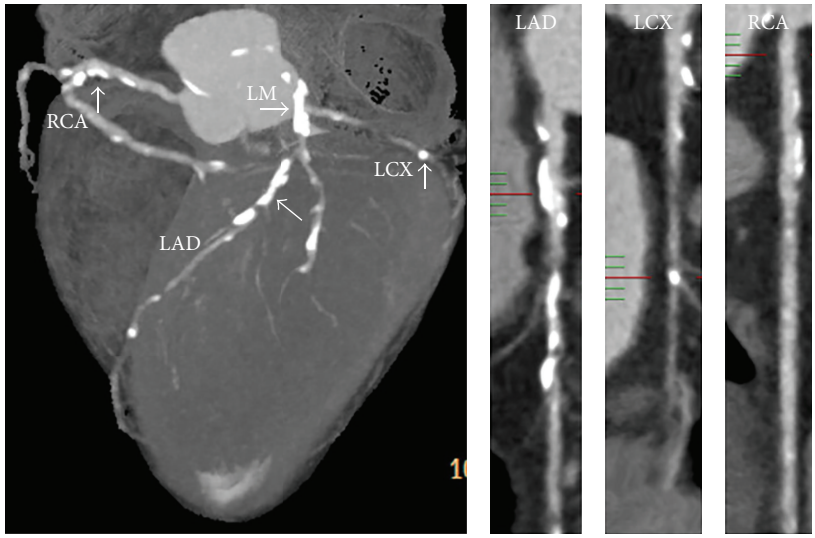

Figure 3: A 62-year-old man with 17 years' history of diabetes with multivessel disease. (a) Volume rendering image depicts unsmooth edges for left and right coronary vessels. (b), (c), and (d) show diffuse calcified plaques and multiple stenosis distributed in the whole course of the LAD, LCX, and RCA arteries (arrows).

values (odds ratio [OR], 2.761; 95% confidence interval [CI], 2.673 to 5.893) and the adjusted model 1 (odds ratio [OR], 3.056; 95% confidence interval [CI], 1.494 to 6.228). No difference was observed in the risk for NCAP or MCAP for the unadjusted values. When examining the presence of a specific plaque subtype, subjects with CRP had no increased risk for the presence of any type of plaque for the adjusted values.

4. Discussion

In the present study, differences in coronary plaque characteristics and plasma hs-CRP levels between symptomatic DM2 patients and nondiabetics were observed. We also demonstrated that elevated hs-CRP levels were associated with increased risk for plaque formation, including CAP and NCAP.

Significantly more diffuse CAP were present in symptomatic DM2 patients compared with nondiabetics, indicating that the presence of diffused calcium is associated with greater atheroma burden, in agreement with previous reports [4, 11]. Traditionally, calcified plaque is considered an established, stable, and quiescent atheroma. However, in several cross-sectional studies in patients with acute coronary syndrome, spotty calcification is associated with culprit lesions [12–14]. Multiple studies also demonstrated that the presence of coronary artery calcification in asymptomatic individuals is a predictor of future cardiac events [15–17]. Clearly, plaque calcification represents a dynamic process related to oxidized lipids and inflammatory activity. The natural history of atherosclerosis is considered a dynamic process varying from early lesion development to more advanced plaques. In our study, we demonstrate that elevated

Table 3: Multivariate logistical regression analysis of coronary plaque subtype and CRP level.

Presence of coronary plaque	Unadjusted OR (95% CI)	Model 1 OR (95% CI)	Model 2 OR (95% CI)
CAP			
Low/normal CRP	1	1	1
Intermediate CRP	1.564 (1.454, 3.284)	2.067 (1.239, 3.961)	1.365 (0.633, 3.925)
High CRP	2.761 (2.673, 5.893)	3.056 (1.494, 6.228)	2.964 (0.129, 4.852)
MCAP			
Low/normal CRP	1	1	1
Intermediate CRP	2.182 (0.715, 6.661)	2.304 (0.739, 7.186)	3.094 (0.678, 14.124)
High CRP	2.727 (0.436, 17.046)	2.266 (0.340, 15.105)	9.043 (0.898, 98.318)
NCAP			
Low/normal CRP	1	1	1
Intermediate CRP	0.397 (0.126, 1.254)	0.422 (0.131, 1.363)	0.358 (0.089, 1.435)
High CRP	0.436 (0.707, 2.727)	0.300 (0.042, 2.143)	2.068 (0.032, 2.255)

Model 1: adjusted for age and sex.
Model 2: adjusted for age, sex, smoking, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, total cholesterol, triglycerides, body mass index, and DM history.

levels of CRP are associated with increased risk for the presence of any plaque and CAP, but not NCAP or MCAP; patients with symptomatic DM2 were more likely to have significant stenosis with CAP in at least one coronary segment. Our findings may indicate a more rapid atherosclerosis development in diabetics, with a faster progression from noncalcified to completely calcified lesions.

Coronary CTA provides data regarding the coronary tree and atherosclerotic plaques beyond simple luminal narrowing and plaque type defined by calcium content [18]. Compared with calcium score analysis, coronary CTA data sets provide submillimeter isotropic spatial resolution, and the possibility of CT attenuation based tissue characterization enables the quantification of total coronary plaque burden and individual plaque components. If the high-risk plaques reported in this study are indeed important for prognosis, coronary CTA would be a potential candidate screening tool, since it might identify silent vulnerable plaques not otherwise detected by functional imaging.

Chronic inflammation plays a major role in all phases of atherosclerosis [9], and DM2 presence and development are associated with subclinical systemic inflammation [19]. In this study, we found that severe stenosis rates and hs-CRP levels were significantly higher in symptomatic DM2 patients. Also, nearly half of the plaques caused obstructive stenosis in symptomatic DM2 patients. It has been clearly demonstrated that both elevated hs-CRP levels and specific plaque subtypes are associated with poor cardiovascular outcomes [10]. Therefore, people at risk should pay more attention to their blood CRP levels. Our findings may help understand how hs-CRP and vulnerable plaques are related, also showing that coronary CTA enables the assessment of such relationship.

Our study has several limitations. First, it remains unclear whether coronary calcification predicts plaque instability or is merely a marker of plaque burden. More animal studies are warranted for better understanding of calcification mechanisms. Second, because we categorized coronary plaque subtypes into NCAP, MCAP, and CAP as opposed to quantifying in a continuous manner the burden of calcified and noncalcified plaque components, there is a chance of misclassification. Finally, although our analysis reflects the relationship between diffused calcification and elevated levels of hs-CRP in patients with diabetes, the resultant impact on clinical outcome remains to be determined. Future studies are required to determine the prognostic role of this methodology in patients with DM2.

In conclusion, combination of coronary CTA and hs-CRP might incrementally improve risk stratification in DM2 patients. Future prospective studies are needed to establish the association between the presence of CAP and acute coronary events.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Jinling Zhang and Zhehao Lv contributed equally to this paper.

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Research Article

The Protective Effect of Low-Dose Ethanol on Myocardial Fibrosis through Downregulating the JNK Signaling Pathway in Diabetic Rats

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Objective. To investigate the effects of low dose ethanol feeding in diabetic rats and analyze its underlying mechanisms. *Methods*. Male Sprague-Dawley rats were divided into 4 groups: control (Con), diabetes at 4 weeks (DM4W), diabetes at 8 weeks (DM8W), and EtOH + DM8W. After 8 weeks, hemodynamic parameters were recorded and heart weight/body weight (H/B) and hydroxyproline (Hp) content in myocardium were measured. Morphology of collagen in myocardial tissue was observed with Masson's trichrome staining method and collagen volume fraction (CVF) was analysed. The mRNA expression of ALDH2 was assessed with Real-Time PCR. The protein expressions of p-JNK and JNK were evaluated using western blot. *Results*. In contrast to Con group, there was no difference in hemodynamic parameters in DM4W group, but mean arterial pressure and heart rate were decreased in DM8W group, and the ratios of H/B, Hp, and CVF were markedly increased. ALDH2 mRNA expression was decreased, while the ratio of p-JNK/JNK were increased. Compared with DM8W group, the above indexes were improved in EtOH + DM8W group. *Conclusion*. With low dose ethanol intervention, enhanced ALDH2 expression can antagonize the happening of myocardial fibrosis in diabetic rats, which may be relevant with downregulating the JNK pathway.

1. Introduction

Diabetes mellitus (DM) is one of the major public health problems around the world [1]. According to the compiled data of the World Health Organization (WHO), approximately 150 million people have diabetes mellitus worldwide, and this number of diabetic patients may be doubled by the year 2025 [2].

Alongside established lifestyle factors, such as smoking, adiposity, and diet, ethanol consumption is thought to play a role in the development of diabetes. Ethanol is a very commonly used chemical substance and also has dose-related effect on cardiac events. Possible cardioprotective effects of ethanol consumption continue to be hotly debated in the medical literatures and popular media [3]. Systematic reviews and meta-analyses have addressed that there is a curvilinear relationship between ethanol consumption and cardiovascular disease, with a protective effect of moderate ethanol consumption and a detrimental effect of large amounts intake [3–7]. Importantly, epidemiological studies suggest that light to moderate alcohol consumption decreases the risk of cardiovascular events, that is, 1-2 drinks per d or 3–9 drinks per wk (one beer, one glass of wine, or one glass of spirit was approximated to one standard drink defined as 1.5 cL or 12 g of pure ethanol [7]). Moderate drinking can activate metabolism of ethanol by acetaldehyde dehydrogenase-2 generation [3] to reduce the incidence of diabetic cardiomyopathy [8, 9].

Diabetic cardiomyopathy is defined as the ventricular dysfunction that occurs in diabetic patients independent of another cause, such as coronary artery disease or hypertension. Diabetic cardiomyopathy is a common complication of diabetes which has become a major cause of diabetes-related morbidity and mortality [10]. Mitochondrial acetaldehyde

dehydrogenase-2 (ALDH2) is a key enzyme which plays an important role in the metabolism of acetaldehyde and other toxic aldehydes [11]. Preclinical studies in rats also suggest that ALDH2 activity may affect diabetic pathology [12]. Furthermore, in transgenic mice, overexpression of ALDH2 has been shown to be protective against streptozotocin-induced diabetic cardiomyopathy [13]. Our previous results had indicated that ALDH2 expression was further decreased accompanying the development of diabetes. Meanwhile, we investigated the effects of low-dose ethanol feeding in diabetic rats which could promote myocardial protection through activation of ALDH2 expression. And more, we also found that upregulation of ALDH2 played a protective effect in myocardial ischemia and reperfusion injury and diabetes cardiomyopathy. ALDH2 may be an endogenous cardiac protective factor in myocardial injury [2, 8, 14].

Several biological mechanisms have been proposed to explain that myocardial fibrosis is one of the main pathological changes of diabetic cardiomyopathy. Studies have found C-JUN N-terminal kinase (JNK) signaling pathway played a key role in the process of myocardial fibrosis [15–17]. Evidence had shown that activation of the JNK pathway was involved in the progression of diabetes induced myocardial fibrosis and that such a pathway could be a therapeutic target for diabetic heart injury and cardiomyopathy [18, 19]. However, whether the JNK pathway is involved in the cardioprotective effect of low-dose ethanol on diabetic rats has not been fully elucidated.

So in this study, we mimic diabetes model by intraperitoneal injection of streptozotocin (STZ) in combination with low-dose ethanol; the purpose of the present study is as follows: (1) to investigate the mechanism of low-dose ethanol which alleviates myocardial fibrosis in diabetic cardiomyopathy; (2) to clarify whether low-dose ethanol mediated protection is associated with downregulating the JNK signaling pathway. This study might shed some light on low-dose ethanol as an effective therapeutic in the treatment of diabetic cardiomyopathy.

2. Methods

2.1. Animals. Adult male Sprague-Dawley rats (200 to 250 g) were obtained from Bengbu Medical College Animal Administration Center. All animal studies were approved by the Animal Ethics Committee of Bengbu Medical College and performed in accordance with the ethical standards. The rats were fed normal chow and had free access to distilled water.

2.2. Chemicals and Reagents. Streptozotocin (STZ) was purchased from Sigma (USA). TRIzol was purchased from Invitrogen (USA); hydroxyproline (Hp) was purchased from Nanjing Jiancheng Bioengineering Institute (China). Ethanol (EtOH) was purchased from Bengbu New Chemical Reagent Factory (China). β-actin antibodies were purchased from Santa Cruz Biotechnology (USA), rabbit c-Jun N-terminal kinase (JNK) and phosphorylated JNK (p-JNK) were purchased from Anbo Biotechnology (USA). Chemiluminescence reaction (ECL) system was purchased from Millipore, Billerica (USA). All primers were purchased from Shanghai Sangon Biotech (China).

2.3. Induction of Diabetes and Experimental Protocol. As previously described by our laboratory, STZ at 55 mg/kg freshly dissolved in 0.1 mol/L sodium citrate buffer (pH 4.5) was injected intraperitoneally to induce diabetic models in overnight fasted rats [8]. All rats were randomly divided into four groups: normal control group (Con), diabetes at 4 weeks group (DM4W), diabetes at 8 weeks group (DM8W), and ethanol + diabetes at 8 weeks group (EtOH + DM8W), respectively ($n = 6$). In Con group, rats were fed with standard rat chow and received an intraperitoneal injection of the same volume of citrate buffer for 8 weeks. In EtOH + DM group, DM rats were fed with 2.5% EtOH in their drinking water for one week to initiate drinking then, it was changed to 5% EtOH continuous access through the remaining 7 weeks.

2.4. Hemodynamics. Male SD rats were anaesthetized by use of chloral hydrate (100 mg/kg) through intraperitoneal injection. Throughout the experiment, systolic pressure (SP), diastolic pressure (DP), mean arterial pressure (MAP), and heart rate (HR) were determined by invasive hemodynamic evaluation methods for 30 min. At the end of the experimental period, hearts were excised rapidly, placed in ice-cold Krebs-Henseleit (K-H) buffer, and weighed and the ratio of heart weight/body weight (H/B) was calculated.

2.5. Detection of Hydroxyproline Content. Heart tissue (100 mg) was homogenized in ice-cold K-H buffer. The supernatant was collected after centrifugation for 20 min (2000 rpm). The protein concentration was measured by the Bradford method. Hydroxyproline (Hp) content was detected according to the instruction manual.

2.6. The Content of Collagen Detection by Masson-Staining. Left ventricular tissue obtained from all groups was stained with Masson's trichrome for the quantification of collagen. The histological sections were taken in 10% neutral formalin-fixed, dehydrated, paraffin embedded sections. Then, the samples underwent the dehydration of gradient ethanol, neutral resin embedding, and Masson trichrome staining. The collagen fibers were stained blue and cardiomyocytes were stained red. Myocardial collagen was quantified at a final magnification of 200x with a polarized microscope connected to a video camera. Myocardial collagen volume fraction (CVF) was analyzed using Image Pro analysis software, expressed as the mean percentage of collagen area to the total area of each microscopic field. Five visions under microscope of each sample were randomly chosen and the average of them was taken for analysis [17].

2.7. Detection of ALDH2 mRNA by Real-Time PCR. Total RNA was extracted from the left anterior myocardium using TRIzol according to the manufacturer's instructions. Total RNA (3 μg) was reversely transcribed to cDNA, and PCR was performed by a routine method. The primers used were as follows: for ALDH2, forward: 5-GTG TTC GGA GAC

Table 1: Hemodynamic data in rats.

Group	SP (mmHg)	DP (mmHg)	MAP (mmHg)	HR (beats/min)
Con	111.09 ± 6.05	95.47 ± 7.75	100.68 ± 7.15	462.63 ± 65.20
DM4W	105.30 ± 4.74$^{\#\#}$	92.76 ± 4.90$^{\#\#}$	96.94 ± 4.85$^{\#\#}$	433.00 ± 14.39$^{\#}$
DM8W	83.24 ± 9.32**	62.97 ± 10.56**	69.72 ± 9.40**	377.06 ± 39.04**
EtOH + DM8W	103.74 ± 4.38$^{\#\#}$	80.45 ± 5.90$^{\#\#}$	88.21 ± 5.37$^{\#\#}$	411.44 ± 22.27*

Values are means ± SD ($n = 6$).
Con: normal control group, DM4W: diabetes at 4 weeks group, DM8W: diabetes at 8 weeks group, and EtOH + DM8W: ethanol + diabetes at 8 weeks group.
$^{*}P < 0.05$, $^{**}P < 0.01$ compared with Con; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with DM8W.

Table 2: Changes of heart weight/body weight (H/B), hydroxyproline (Hp) content, and collagen volume fraction (CVF) in different groups.

Group	H/B (mg/g)	Hp (umol/mg)	CVF (%)
Con	3.76 ± 0.15	0.27 ± 0.05	12.50 ± 0.98
DM4W	3.88 ± 0.20$^{\#\#}$	0.35 ± 0.07	15.74 ± 1.53$^{*\#\#}$
DM8W	4.78 ± 0.10**	0.43 ± 0.04**	25.75 ± 1.98**
EtOH + DM8W	3.96 ± 0.17$^{\#\#}$	0.34 ± 0.05$^{\#}$	14.43 ± 2.65$^{\#\#}$

Values are means ± SD ($n = 6$).
$^{*}P < 0.05$ and $^{**}P < 0.01$ compared with Con; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with DM8W.

GTC AAA GA-3′ and reverse: 5′-GCA GAG CTT GGG ACA GGT AA-3′ and the product size was 187 bp; for β-actin, forward: 5′-GAG ACC TTC AAC ACC CCA GCC-3′ and reverse: 5′-GGC CAT CTC TTG CTC GAA GTC-3′ and the product size was 312 bp [8]. The PCR condition was as follows: predenaturing at 95°C for 3 min and then 40 cycles (50 s denaturation at 95°C, 50 s annealing at 62.5°C, and 60 s extension at 72°C), followed by a final step at 72°C for 10 min. Real-Time PCR was performed to determine the ALDH2 mRNA level.

2.8. Detection of JNK and p-JNK Protein Expressions by Western Blot. Myocardium tissues (100 mg) from each group were collected and homogenized in a lysis buffer. Homogenates were sonicated and centrifuged at 12,000 ×g for 30 min at 4°C. The protein concentration was determined using the bicinchoninic acid (BCA) Protein Assay kit. Total protein (80 μg) was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred electrophoretically to a polyvinylidene difluoride (PVDF) filter membrane [20].

The membranes were blocked with 5% nonfat milk in Tris-buffered saline Tween (TBST) for 2 h, and then they were incubated at 4°C overnight with the corresponding primary rabbit JNK antibody (1 : 1000), rabbit p-JNK antibody (1 : 1000), and mouse β-actin antibody (1 : 500). All membranes were incubated for 1 h with corresponding secondary antibody HRP-linked anti-mouse IgG or HRP-linked anti-rabbit IgG. Autoradiographs were scanned and the band density was determined with Image J software.

2.9. Statistical Analysis. Data were expressed as mean ± SD. One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) was used for multiple comparisons. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Hemodynamics. In contrast to Con group, there was no statistical difference about hemodynamic parameters in DM4W groups, but systolic pressure (SP), diastolic pressure (DP), mean arterial pressure (MAP), and heart rate (HR) were decreased significantly in DM8W groups. Compared with DM8W group, SP, DP, MAP, and HR were rather increased significantly in EtOH + DM8W group. (Table 1)

3.2. The Ratio of Heart Weight to Body Weight and Hydroxyproline Content in Myocardial Tissue. Compared with Con group, there was no significant difference about the ratio of heart weight to body weight (H/B) and hydroxyproline (Hp) content in myocardial tissue in diabetic rat in DM4W group; however, with extended duration, H/B ratio and myocardial Hp content were increased in DM8W group, and the difference was statistically significant (Table 2). Compared with DM8W group, H/B ratio and myocardial Hp content were significantly deceased in EtOH + DM8W group ($P < 0.05$).

3.3. The Content of Collagen Detection by Masson-Staining. In Masson trichrome staining, the collagen fibers were stained blue and cardiomyocytes were stained red. The collagen tissue was appropriately arranged among cardiomyocytes in control group. However, collagen tissue was increased markedly and disrupted in some area in diabetic group.

In DM4W group, myocardial cells were approximately arranged well, collagen fibers were sparsely distributed, and interstitial collagen was dyed a little blue. Compared with control group, myocardial cells were in a disordered arrangement, the interstitial collagen were edematous, and collagen fibers were unevenly distributed and increased markedly in DM8W group. Compared with DM8W group, myocardial cells were arranged neatly, and collagen fibers were significantly reduced in EtOH + DM8W group.

Quantitative Analysis Results. The content of collagen in diabetic group was higher than that of control group. As displayed in Table 2, compared to control group, the contents of collagen volume fraction (CVF) were significantly increased in DM4W group ($P < 0.05$), and with the development

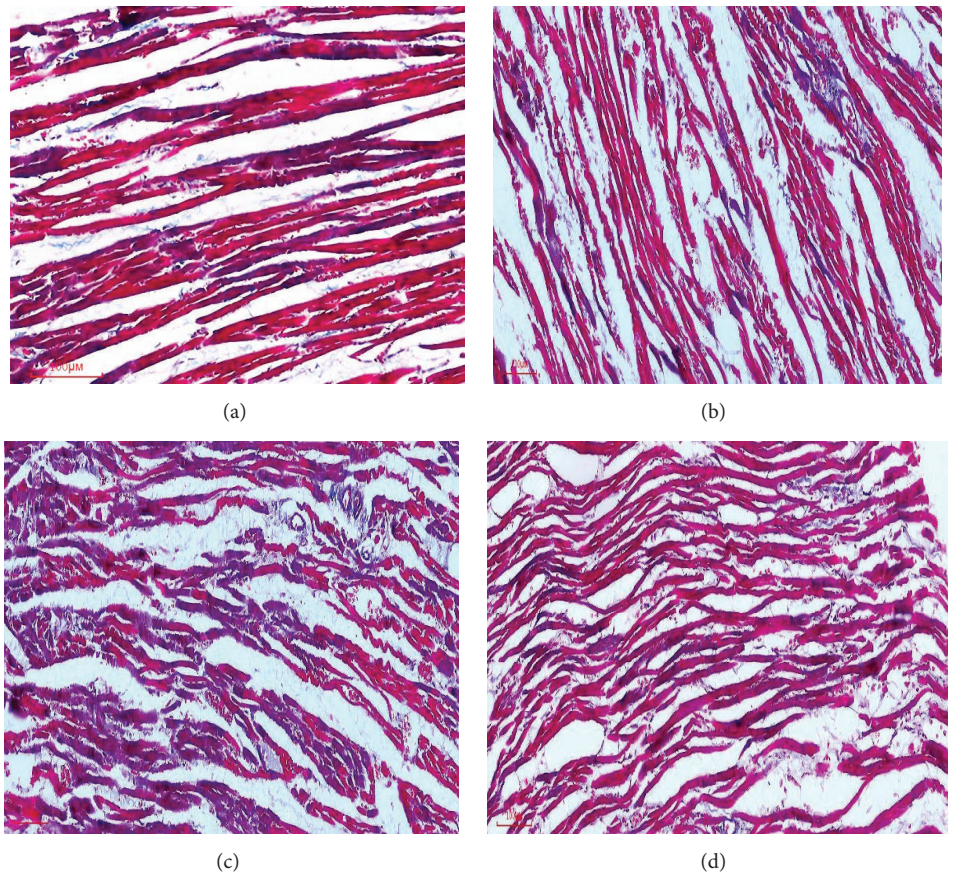

Figure 1: The result of collagen detection by Masson-staining (200). (a) Control, (b) DM4W, (c) DM8W, and (d) EtOH + DM8W.

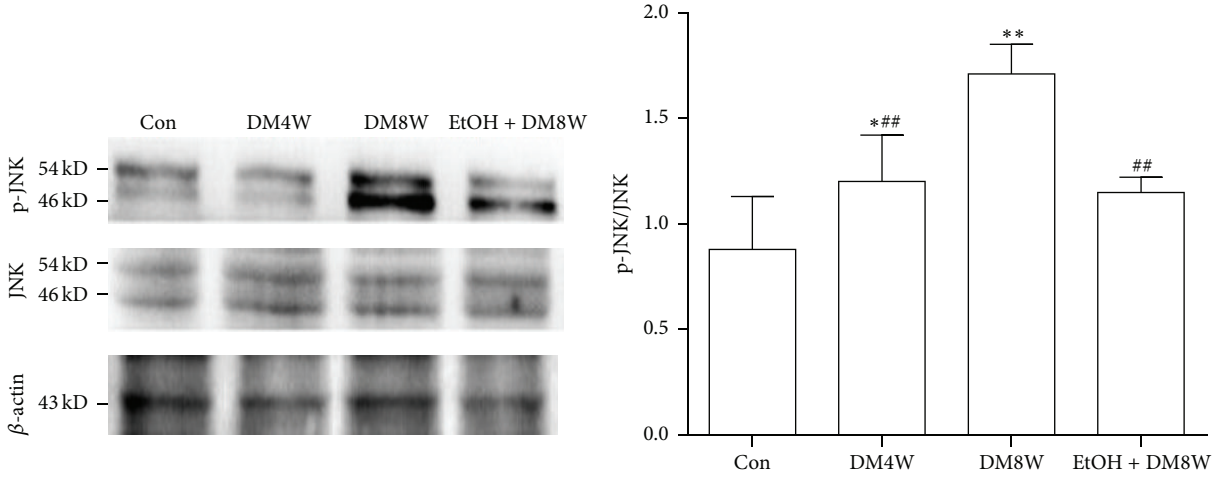

Figure 2: The result of myocardial JNK and phospho-JNK protein: $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with Con; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with DM8W.

of diabetes, CVF were further increased in DM8W groups ($P < 0.01$). But in contrast to DM8W group, the contents of CVF were markedly decreased in EtOH + DM8W group ($P < 0.01$, Figure 1, Table 2).

3.4. Changes of Myocardial ALDH2 mRNA Expression. Real-Time PCR revealed that, compared with control group, the expression of ALDH2 mRNA level was reduced in DM4W ($P < 0.05$). With the development of diabetes, ALDH2 mRNA were further decreased in DM8W groups ($P < 0.01$). In contrast to DM8W group, the expression of ALDH2 mRNA was increased in EtOH + DM8W group ($P < 0.01$, Table 3).

3.5. Changes of Myocardial JNK and Phospho-JNK Protein Levels. On western blot analysis, compared with control

Table 3: The result of ALDH2 mRNA and p-JNK/JNK protein expression.

Group	ALDH2 mRNA	p-JNK/JNK
Con	0.85 ± 0.16	0.88 ± 0.25
DM4W	$0.56 \pm 0.17^{*\#\#}$	$1.20 \pm 0.22^{*\#\#}$
DM8W	$0.21 \pm 0.0^{**}$	$1.71 \pm 0.14^{**}$
EtOH + DM8W	$0.61 \pm 0.18^{\#\#}$	$1.15 \pm 0.07^{\#}$

Values are means ± SD ($n = 6$).
$^{*}P < 0.05$ and $^{**}P < 0.01$ compared with Con; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with DM8W.

group, the ratio of p-JNK/JNK protein expression was increased in DM4W ($P < 0.05$), and with the development of diabetes, the ratio of p-JNK/JNK was further increased in DM8W groups ($P < 0.01$). Compared with DM8W group, the ratio in EtOH + DM8W group was decreased ($P < 0.01$, Figure 2, Table 3).

4. Discussion

Diabetes mellitus is becoming an epidemic health threat and represents one of the most prevalent chronic noncommunicable disorders [21]. Cardiovascular disease is a serious complication of diabetes and is responsible for 80% of the deaths among diabetics [22]. Diabetic cardiomyopathy is one of the most common complications of diabetes; its major pathological characteristics are hypertrophy or hyperplasia of cardiac myocytes. Excessive deposition of myocardial interstitial collagen and myocardial fibrosis often leads to cardiac hypertrophy and decrease of heart function, which play a vital role in the occurrence and development of diabetic cardiomyopathy [23].

In the present study, our results demonstrated that, with the progression of diabetes, systolic pressure, diastolic pressure, mean arterial pressure, and heart rate were declined significantly in DM8W group compared with Con group, but the ratio of heart weight/body weight, hydroxyproline content, and CVF were markedly increased. Meanwhile, the mRNA expression of ALDH2 was decreased and the ratio of p-JNK/JNK was increased. When the DM rats were treated with ethanol at low concentration, hemodynamic parameters were improved and hydroxyproline content and CVF were decreased, accompanied with the increase of myocardial ALDH2 mRNA expression and decrease of p-JNK/JNK protein expression. The results suggested that with low-dose ethanol intervention, enhanced ALDH2 expression can antagonize the happening of myocardial fibrosis in diabetic cardiomyopathy, which may be relevant with downregulating the JNK pathway.

Mitochondrial aldehyde dehydrogenase 2 (ALDH2) is a kind of aldehyde oxidase that is involved in ethanol metabolism, which is closely related to human alcohol drinking behavior [24]. Heavier alcohol consumption is associated with increased risk of developing diabetes mellitus, hypertension, and cardiovascular and cerebrovascular disease [25]. Nevertheless, there are increasing reports showing a critical role for light to moderate alcohol ingestion could protect against myocardial injuries. Researches indicated a U-shaped relationship between alcohol consumption and cardiovascular disease mortality [6, 26]. Statistics manifested the mortality of drinking alcohol up to or over 6 cups (355 mL/cup) per day is 1.6 times as high as control, while light-to-moderate drinking of 1~2 cups alcohol per day [fewer than 2 cups or 1 ounce (28.3495 g)] reduced mortality from cardiovascular disease [27]. In addition, clinical data showed that moderate drinking can protect metabolic syndrome (cardiovascular disease and diabetes) via influencing various factors to reduce the diabetes prevalence, such as glycosylated hemoglobin, high density lipoprotein cholesterol, and fibrinogen [28]. After continuously feeding C57BL/6 mice with 18% of ethanol for 12 weeks, the myocardial mechanics were restored and the expression and activation of PKC and Akt were increased, which established that EtOH feeding causes cardiac expression of activated PKC-ε [29]. Sun et al. observed that ischemic injury could result in downregulation of mitochondrial ALDH2 in mice hearts inducing an elevation of aldehyde 4-hydroxy-2-nonenal (4-HNE), leading to cardiomyocyte apoptosis through downregulation of HSP70 and activation of JNK and p53. ALDH2 detoxifies 4-HNE, a mediator of programmed cell death events, by transmitting a mitochondrial ALDH2 signal to elicit a cytosolic response through the JNK/p53 pathway. However, using transgene technology to increase the expression of ALDH2 can contribute to antagonizing heart failure and decreasing heart function [30]. Furthermore, our previous research found that there was a close relationship between ALDH2 and oxidative damage. With the progression of diabetes, the myocardial antioxidant ability of diabetic rats was decreased, which exacerbated the progression of myocardial fibrosis [8, 13, 31]. In this study, we found that the ratio of heart weight/body weight, hydroxyproline content, and CVF were markedly increased in diabetic 4 and 8 weeks' rats, which suggested diabetic induced myocardial fibrosis. When the diabetic rat was treated with low doses of ethanol to induce ALDH2 activity, the hemodynamic parameters were increased and hydroxyproline content and CVF were decreased, which indicated that increasing ALDH2 expression can attenuate the happening of myocardial fibrosis and the destroying of myocardial injuries.

Mitogen-activated protein kinases (MAPKs) play an important role in the signal transduction pathways from the membrane to intracellular compartments including the nucleus. They regulate the functions of many gene products and therefore affect cell growth, differentiation, and apoptosis [32]. Oxidative stress, inflammation, endoplasmic reticulum stress, and autophagy defect can activate MAPKs signaling pathway in the progression of diabetes complications. Li et al. observed that production of a large number of ROS was thought to be an important contributing factor, concomitant with activation of JNK, p38 MAPK, and TGF-β in the development and the progression of diabetic cardiomyopathy [16].

C-Jun NH2-terminal kinase (JNK) is one of the major members of MAPKs, and JNK activation is also implicated in cardiac fibrosis [33, 34]. JNK signaling pathway plays a key role in the growth of cardiac fibroblasts induced by

high glucose. Studies of diabetic rats had demonstrated that myocardial fibrosis was developed; meanwhile, JNK mRNA expression level and activity were upregulated [17]. Furthermore, disruption of the JNK protein kinase decreased the occurrence and development of diabetic myocardial fibrosis [18, 35]. It is worthwhile to note that, in our experiment, we found that, with the progression of diabetes, the expression of myocardial ALDH2 at mRNA level was decreased and the ratio of p-JNK/JNK at protein level was increased, which suggested ALDH2 and JNK signaling pathway both participated in the occurrence and development of diabetic cardiomyopathy. Moreover, further enhancing activation of ALDH2 expression of low doses of ethanol for 8 weeks in diabetic rats, accompanied with the high-expression of ALDH2, p-JNK/JNK were decreased in contrast to diabetes rats, which suggested activation of ALDH2 expression might be associated with downregulating the JNK signaling pathway to relieve myocardial fibrosis and myocardial injuries.

However, there were some experimental limitations in our study. We only observed that the protection of low-dose ethanol was relevant with downregulating the JNK pathway in diabetic cardiomyopathy. To better understand the mechanisms involved, we will adopt the activation or inhibition of JNK to investigate the downstream signaling molecules in the follow-up experiments. To address this issue, we will use JNK inhibitor to observe whether inhibiting JNK pathway can play the cardiovascular role in diabetes rats. Moreover, we further explore whether enhanced activation of JNK signaling pathway can antagonize the cardioprotective effect of activation of ALDH2 by low-dose ethanol. Through these experiments, we want to verify low-dose ethanol could attenuate myocardial fibrosis via downregulating the JNK pathway in diabetic cardiomyopathy.

In summary, our results demonstrate that with the progression of diabetes, ALDH2 expression was decreased accompanied with the happening of myocardium fibrosis. Treatment with ethanol at low concentration can protect the heart by upregulating ALDH2 and downregulating the JNK signaling pathway against myocardial fibrosis in diabetic cardiomyopathy.

Competing Interests

The authors have no potential conflict of interests to declare.

Authors' Contributions

Ying Yu and Xian-Jie Jia are co-first authors and contributed equally to this paper.

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Review Article

The Role of AGE/RAGE Signaling in Diabetes-Mediated Vascular Calcification

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AGE/RAGE signaling has been a well-studied cascade in many different disease states, particularly diabetes. Due to the complex nature of the receptor and multiple intersecting pathways, the AGE/RAGE signaling mechanism is still not well understood. The purpose of this review is to highlight key areas of AGE/RAGE mediated vascular calcification as a complication of diabetes. AGE/RAGE signaling heavily influences both cellular and systemic responses to increase bone matrix proteins through PKC, p38 MAPK, fetuin-A, TGF-β, NFκB, and ERK1/2 signaling pathways in both hyperglycemic and calcification conditions. AGE/RAGE signaling has been shown to increase oxidative stress to promote diabetes-mediated vascular calcification through activation of Nox-1 and decreased expression of SOD-1. AGE/RAGE signaling in diabetes-mediated vascular calcification was also attributed to increased oxidative stress resulting in the phenotypic switch of VSMCs to osteoblast-like cells in AGEs-induced calcification. Researchers found that pharmacological agents and certain antioxidants decreased the level of calcium deposition in AGEs-induced diabetes-mediated vascular calcification. By understanding the role the AGE/RAGE signaling cascade plays diabetes-mediated vascular calcification will allow for pharmacological intervention to decrease the severity of this diabetic complication.

1. Introduction

Diabetes mellitus is a family of diseases characterized by elevated blood glucose levels or hyperglycemia resulting from the body's inability to produce and/or use the insulin hormone. Type I diabetes mellitus is associated with pancreatic β cell dysfunction resulting in the loss of insulin production, whereas type II diabetes mellitus is caused by insulin receptor dysfunction in which insulin receptor signaling is uncoupled from glucose uptake. Diabetes mellitus is highly prevalent in the United States with approximately 29 million people living with diabetes or 9.3% of the population [1]. It is reported that the death rate from cardiovascular disease for an individual, 18 years and older, with diabetes was about 1.7 times higher than the normal population [1]. Increased death rates from diabetic cardiovascular disease demonstrate the severity of the complications that can arise from this pathology. Therefore, the link between cardiovascular disease and diabetes is essential to understand [2].

2. Type II Diabetes and Vascular Calcification

Type II diabetes has been heavily linked to vascular calcification through several different mechanisms, some of which include oxidative stress, hyperglycemia, hyperkalemia, and hypercalcemia with oxidative stress being the main focus of this review [3–5]. Vascular calcification is described as the hardening of the medial layer of the artery through deposition of hydroxyapatite minerals into the extracellular matrix [6–8]. This process, once thought to be passive and associated with aging, has now been demonstrated to be a tightly regulated cell-mediated process [3]. During vascular calcification, bone morphogenetic protein-2 (BMP-2) activates core binding factor alpha-1 (CBFA-1, also known as RunX2), which acts as the primary transcriptional regulator for the maturation of osteoblasts in the bone [9–11]. CBFA-1 also upregulates the production of osteoblast proteins within vascular smooth muscle cells (VSMCs), which is thought to cause a phenotypic switch of VSMCs to an osteoblast-like phenotype [12]. Alkaline phosphatase (ALP)

and bone sialoprotein (BSP) have been demonstrated to be early markers of osteoblast activity, while markers, such as osteopontin (OPN) and osteocalcin, are upregulated late in the calcification process [13–15]. Their primary function is to enhance the formation and deposition of hydroxyapatite, which is composed of type I collagen and other noncollagenous proteins [15]. Primarily indicated in bone formation, ALP is responsible for cleaving pyrophosphate to phosphate to promote hydroxyapatite deposition and mineralization within the bone [16]. BSP is responsible for the nucleation of hydroxyapatite mineral [15, 17, 18]. Similar to ALP, OPN is also linked to hydroxyapatite deposition and can serve as a mediator of cell attachment and signaling [19]. Hydroxyapatite size and shape are mediated by osteocalcin through a vitamin K dependent mechanism [20]. Taken together, these data demonstrate the potential to promote bone formation within a living system, and researchers have utilized this knowledge of bone matrix proteins to understand the underlying mechanisms of vascular calcification and type II diabetes.

In a series of studies performed by Chen et al., arteries harvested from diabetic and nondiabetic patients were analyzed to determine the amount of calcium, OPN, ALP, type I collagen, and BSP. With the exception of BSP, all investigated bone matrix proteins were significantly increased as a result of diabetes [21]. *In vitro* experiments, using bovine vascular smooth muscle cells (BVSMCs) grown in euglycemic (normal glucose) and hyperglycemic conditions, revealed that CBFA1, ALP, and osteocalcin levels were significantly higher in cells grown in a high glucose media. In addition, calcium deposition was also significantly higher in high glucose than in normal glucose media, and this trend was also observed when both types of growth media conditions were supplemented with calcification media. Calcification media contain elevated levels of inorganic phosphate to promote calcification through utilization of the cells that need to maintain homeostasis. To determine the signaling mechanisms responsible for the increased bone matrix protein expression, BVSMCs were exposed to high glucose levels and protein kinase C (PKC) activity was pharmacologically inhibited in both normal and high glucose treated cells. PKC was selected as the signaling pathway focus due to its predetermined role in cellular responses to diabetes and hyperglycemia [22, 23]. As a result, the expression of bone matrix proteins was significantly decreased, whereas, in normal glucose treated cells, there was no notable change in protein expression. This study also demonstrated enhanced BMP-2 secretion from BVSMCs cultured in high glucose media. Overall, Chen et al. concluded that hyperglycemic conditions, as observed in diabetes, promoted the upregulation of bone matrix proteins and vascular calcification [21, 24]. Supporting studies by Mori et al. demonstrated OPN was upregulated and activated by a similar PKC-mediated pathway in diabetic rat VSMCs. Western blotting confirmed that PKC inhibition resulted in a notable decrease in OPN protein expression [25–27]. Taken together, these studies have shown not only the prevalence of bone matrix protein expression in vascular smooth muscle cells but also the role of PKC in diabetes-mediated vascular calcification.

3. Vascular Calcification and AGE-RAGE Signaling

In addition to increased bone matrix protein expression in VSMCs during diabetic and calcification treatments, studies have also shown that advanced glycation end products (AGEs) and their receptors (RAGEs) play a role in vascular calcification [28]. Type II diabetes patients have been shown to have a significantly higher concentration of AGEs than the nondiabetic population [29–31]. AGEs form over a lifetime as a result of increased circulating glucose as well as other reducing sugars, such as galactose and fructose, reacting with amino groups of proteins to form Schiff bases to either follow the polyol pathway to yield AGEs or be degraded [32]. These glycated end products interact with RAGEs, which are transmembrane proteins that are a part of the immunoglobulin superfamily. RAGEs are upregulated in response to increased circulating AGE levels [33]. Upon AGE-RAGE binding, RAGE works through PKC-ζ to trigger the downstream activation of a signaling cascade that works through p38 mitogen activated protein kinase (MAPK), transforming growth factor-β (TGF-β), and nuclear factor κB (NFκB) [34, 35]. Suga et al. demonstrated that activation of the AGE-RAGE signaling in rat VSMCs reduced the expression of VSMC gene markers such as smooth muscle-myosin heavy chain (SM-MHC) and smooth muscle 22α (SM22α) [36]. This downregulation of VSMCs markers suggests the possible phenotypic switch of VSMCs to an osteoblast-like phenotype [12]. This is supported by findings from human VSMCs (HVSMCs) where activation of RAGE increased mRNA expression and activity of ALP, a bone matrix protein, suggesting a role for RAGE signaling in vascular calcification [36]. These studies demonstrated some basic roles for RAGE in VSMC calcification through PKC-ζ signaling, increased expression of ALP, and decreased expression of VSMC gene markers.

In studies performed by Tanikawa et al., using an HVSMC *in vitro* calcification model increasing the levels of AGEs significantly increased the amount of calcium deposition after 7 and 14 days when compared to BSA treated and control samples [37]. Additionally, mRNA expression of CBFA-1 (RunX2), ALP activity, and osteocalcin protein levels were also significantly elevated. Together, these data indicated that AGE treatment promotes an osteoblast-like phenotype in HVSMCs. This phenotypic switching was not dependent on calcification media as similar results were found using HVSMCs grown with and without calcification media [21]. VSMC expression of osteoblast proteins may be linked to p38 MAPK activity as Tanikawa et al. found that, with increased AGE exposure, p38 MAPK activation was increased. Conversely, when RAGE signaling was dampened, p38 MAPK activation was decreased, and the changes in p38 MAPK correlated to decreased levels of ALP activity despite AGE-induced calcification [37]. In a similar study by Hu et al., p38 MAPK was shown to be essential for osteoblast differentiation in MC3T3-E1 cells. Pharmacological inhibition of p38 MAPK resulted in decreased in ALP activity, thus, demonstrating that p38 MAPK is required for ALP expression in osteoblast-like cells [38]. Therefore, ALP

activity can be directly influenced by both increased AGE exposure and elevated RAGE cascade signaling through p38 MAPK. This relationship suggests that p38 MAPK plays a key role in the AGE-RAGE pathway in diabetes-mediated vascular calcification [37].

While these findings demonstrate the importance of the AGE-RAGE pathway in diabetes-mediated vascular calcification, Ren et al. demonstrated that AGEs also significantly increased intracellular calcium levels in rat VSMCs [37, 39, 40]. It was found that mRNA levels of ALP and OPN were significantly increased after a 24-hour exposure to glycated albumin (AGE-BSA). Due to the increase in ALP and OPN with AGE-BSA treatment, the group also demonstrated that RAGE was upregulated in the rat VSMCs. When incubated with a neutralizing antibody to RAGE, the amount of calcium and ALP expression was decreased. The observed changes confirmed that RAGE mediates AGE-induced VSMC calcification [39]. Wei et al. showed that diabetes accelerated aortic calcification in male Wistar rats [41]. The animals were treated with streptozotocin (STZ) to induce diabetes and then treated with Vitamin D3 and nicotine (VDN) to induce vascular calcification. von Kossa staining allowed for visualization of the calcium particles within the removed aortic tissue, and calcium particles were found within the selected tissue section. Western blot analysis showed a significant increase in ALP expression and the levels of AGEs were also increased in the diabetic and VDN treated animals [41]. It is important to point out that while AGE-RAGE signaling can directly mediate vascular calcification in diabetes, AGE-RAGE signaling can also indirectly impact this diabetic complication.

4. Roles for Fetuin-A in Vascular Calcification and RAGE Signaling

Serum protein α_2-Heremans-Schmid glycoprotein (Ahsg or fetuin-A), a systemically circulating glycoprotein, has been implicated in insulin resistance in type II diabetic patients [42]. Patient data revealed that high levels of serum fetuin-A were an indicator for hyperglycemia in type II. Fetuin-A also hindered insulin reception through inhibition of the insulin receptor to autophosphorylate insulin receptor substrate-1 protein, which is crucial to the insulin receptor signaling pathway [43, 44]. Collectively, these studies revealed that fetuin-A plays a role in insulin resistance in type II diabetes which can lead to further exacerbation of hyperglycemia and other diabetic complications. Interestingly, increased levels of vascular calcification have been demonstrated to be associated with not only type II diabetes but also patients with chronic kidney disease (CKD) [45]. Vascular calcification, in this instance, has been shown to promote both inflammatory and oxidative stress response to compound it as a risk factor for cardiovascular disease. Fetuin-A is released by the liver to function as an acute phase protein in the innate immune system where it functions to promote anti-inflammatory and antioxidative stress responses to inhibit overexpressed inflammatory molecules.

Conversely, fetuin-A can also elicit an innate immune response elicited in part by toll-like receptors (TLRs). This mechanism can be activated by free fatty acids (FFAs) to induce a proinflammatory response [46]. Pal et al. showed that fetuin-A can act as a ligand to TLR-4 to stimulate FFA-induced insulin resistance in adipocytes [47]. In addition to promoting insulin resistance in type II diabetic patients, fetuin-A can also inhibit an alternate RAGE ligand, high mobility group box-1 (HMGB1), which is responsible for the release and recruitment of several cytokines, adhesion molecules, and chemokines. RAGE signal cascade activation has been demonstrated to be responsible for HMGB1 mediated expression of tumor necrosis factor (TNF) and interleukin-1 (IL-1) [48, 49]. Of concern, fetuin-A inhibition of HMGB1 could possibly create a setting for RAGEs to preferentially select and bind AGEs to activate the cascade. Using data collected from CKD patient samples, Janda et al. demonstrated that increased serum fetuin-A levels were a positive indicator for increased deposition of AGEs within the arteries, thus, indicating that fetuin-A may indirectly influence the AGE/RAGE pathway especially in the presence of inflammatory molecules.

Fetuin-A (Ahsg) has a high affinity for hydroxyapatite crystals, which are located in sites of vascular calcification, such as bone and teeth [45, 50, 51]. Ketteler et al. utilized patients with CKD on hemodialysis to correlate cardiovascular mortality with decreased fetuin-A levels and increased vascular calcification suggesting that fetuin-A acts as an inhibitor of calcification [6, 52, 53]. Studies using a fetuin-A deficient mice model that were calcification sensitive (DBA/2-Ahsg$^{-/-}$) determined that the glycoprotein is an inhibitor of calcification [54]. X-ray images of the bone and von Kossa staining of the lung, heart, kidney, and skin revealed a visual increase in the deposition of phosphorus and calcium in each tissue type. Blood serum was extracted from DBA/2-Ahsg$^{-/-}$ animals to perform an *in vitro* basic calcium phosphate (BCP) precipitation assay. Fetuin-A decreased the amount of BCP precipitate within the serum, indicating that fetuin-A can inhibit the formation of BCP deposition [54]. Within the same research group, Heiss et al. utilized electron microscopy and dynamic light scattering to determine the structural characteristics of fetuin-A complexing with BCP to form calciprotein particles. Additional studies using purified fetuin-A incubated with BCP *in vitro* resulted in BCP structure changing from a rigid to a fragile appearance [3, 55]. This observed structural change was also observed in other calcium based materials such as $CaCO_3$ nanoparticles [56].

The relationship between fetuin-A, BCP, and calcified VSMCs was determined using *in vitro* and *in vivo* HVSMCs model system. Reynolds et al. demonstrated that fetuin-A was localized in the matrix vesicles of calcified HVSMCs in the medial layer of the artery [57]. These calcified HVSMCs were treated with fetuin-A, which inhibited calcium deposition and calcium incorporation in a dose-dependent and cell-mediated manner. VSMCs have been shown to undergo vesicle- and apoptotic body-mediated vascular calcification [58, 59]. Microscopy and western blotting revealed that HVSMC apoptosis was inhibited by fetuin-A. The calcification of released matrix vesicles and apoptotic bodies was quantified by energy dispersive X-ray analysis and showed

that fetuin-A also inhibits calcification of these released cell particles. In this same study, it was demonstrated that fetuin-A is an inhibitor of HVSMC calcification mediated by matrix vesicles and apoptotic bodies [57]. In similar studies by Moe et al., fetuin-A was shown to be an inhibitor of calcification in BVSMCs [60, 61]. Taken together, these data demonstrate that fetuin-A is an inhibitor of calcification.

5. AGE-RAGE Signaling and Oxidative Stress in Vascular Calcification

The AGE/RAGE signaling cascade has been demonstrated to be akin to a feed-forward loop whereby outcomes such as increased fibrosis, increased RAGE expression, and increase oxidative stressors are produced [62, 63]. Oxidative stress produced by elevated reactive oxygen species (ROS) can disrupt numerous intracellular structures, such as cellular membranes, proteins, lipids, and DNA. ROS products, like hydrogen peroxide, superoxide anions, hydroxyl radicals, and nitric oxide, are generated by mitochondrial oxidases, NADPH oxidases (Nox), and nitric oxide synthases [64]. RAGE activation results in the increased production of ROS by stimulating specific signaling cascades such as TGF-β, NF-κB, and Nox-1 [62]. In a study performed by Wei et al., malondialdehyde (MDA) concentration and Cu/Zn superoxide dismutase (SOD-1) activity were used to assess oxidative stress and the ability to initiate a compensatory oxidative stress mechanism in diabetes-mediated vascular calcification animal models. Diabetic animals with VDN-induced vascular calcification had a significant increase in MDA content and significant decrease in SOD activity levels compared to the diabetic group. When isolated VSMCs were treated with increasing levels of AGE, there were elevated ALP activity levels, Nox-1 mediated ROS production, and RAGE expression. Inhibiting RAGE expression consequently decreased ALP activity, calcium content, and Nox-1 protein production while simultaneously increasing SOD-1 levels. Overall, these studies demonstrated that cell isolates from a model diabetes with VDN mediated vascular calcification model were respondent to AGE treatments as evidenced by significantly increased levels of ALP, ROS, Nox-1, and RAGE protein when compared to only diabetic animals [41]. Brodeur et al. utilized a similar animal model to determine if AGEs within an *in vivo* system can be reduced after diabetes-mediated vascular calcification has occurred [65]. Pyridoxamine (PYR), an AGE inhibitor, was administered as a preventive precalcification treatment whereas alagebrium (ALA), an AGE breaker, was given as a therapeutic postcalcification treatment. For these studies, only ALA allowed for a significant reduction in the number of AGEs and calcium content as measured in muscular arteries, such as the femoral artery, but not in larger conducting arteries like the aorta. PYR decreased the overall AGE and calcium levels, but it was not significant in the studied tissues. The difference in effectiveness of both treatments could be due to the mechanisms of action; PYR acts as an AGE preventative whereas ALA acts as an AGE crosslink breaker. The efficacy of several antioxidants therapies, such as alpha-lipioc acid, 4-hydroxy tempol, and apocynin, was also tested. Apocynin treatment resulted in a significant reduction in calcium deposition in the diabetes-mediated vascular calcification animal model. Brodeur et al. demonstrated that a reduction in calcium through targeted ROS antioxidant therapy is a more feasible treatment in an *in vivo* model of vascular calcification [65]. Collectively, these studies demonstrate that the AGE/RAGE cascade is capable of mediating vascular calcification through oxidative stress mechanisms, and therapeutic treatments to limit ROS production might provide a more feasible alternative to minimize vascular calcification.

Another ROS signaling cascade activated by AGEs is transforming growth factor- (TGF-) β. In a study by Li et al. when VSMCs were treated with AGEs, members of the AGE/RAGE signaling cascade (i.e., p38 MAPK and ERK1/2) were found to be phosphorylated upon RAGE activation [66]. In addition, TGF-β signaling resulted in the phosphorylation of its family of mediators, Smads, which serve as transcriptional modulators [67]. These changes were found to be TGF-β dependent. Western blot analysis revealed that when RAGE expression was downregulated, Smad 2 phosphorylation was also inhibited indicating the AGE/RAGE cascade in Smad activation and TGF-β signaling. Since the accumulation of AGEs is within the extracellular matrix (ECM), it is important to note that an increase in TGF-β has been implicated in fibrosis within disease [68]. Fibrosis is typically associated with an increase in type I collagen and Li et al. utilized western blot analysis to demonstrate that AGEs induce an increased production of type I collagen, which was inhibited by blockade of p38 MAPK and ERK1/2 signaling. These data allow for the conclusion that AGE/RAGE signaling plays a role in the maintenance and regulation of the ECM in diabetes and that AGEs induce TGF-β through mediation by RAGE [63, 66, 68].

AGEs have also been shown to increase the activity of NFκB through RAGE signaling in VSMCs. Studies have demonstrated that VSMCs will maintain a compliant, contractile phenotype within the artery; however, increases in NFκB signaling will interfere with this phenotype resulting in increased rigidity and stiffness commonly associated with cardiovascular diabetic complications [69]. Simard et al. treated rat aortic VSMCs (A7r5 cells) with glycated human serum albumin (AGE-HSA) and using GFP expression observed significantly increased NFκB activity. Western blot analysis revealed that ERK1/2 activation was significantly increased with AGE-HSA treatment, and AKT activation was slightly increased. Both of these pathways activate NFκB, which would allow for the conclusion that RAGE signaling increases NFκB activity [69]. An increase in NFκB transcription activity can lead to an increase in mRNA expression of type I collagen a1 and a2 in murine VSMCs treated with AGEs as shown in Peng et al. [70]. Collectively, AGE-induced RAGE signaling affects the activity of NFκB in VSMCs, which can lead to remodeling of the type I collage in the ECM or to a change in cell morphology. Also, when they are treated with AGE-HSA, the mRNA levels of smooth muscle-myosin heavy chair (SM-MHC) and SM-22α were decreased, and additionally protein expression of SM-α-actin, SM-22α, and myocardin (MyoC) was also decreased.

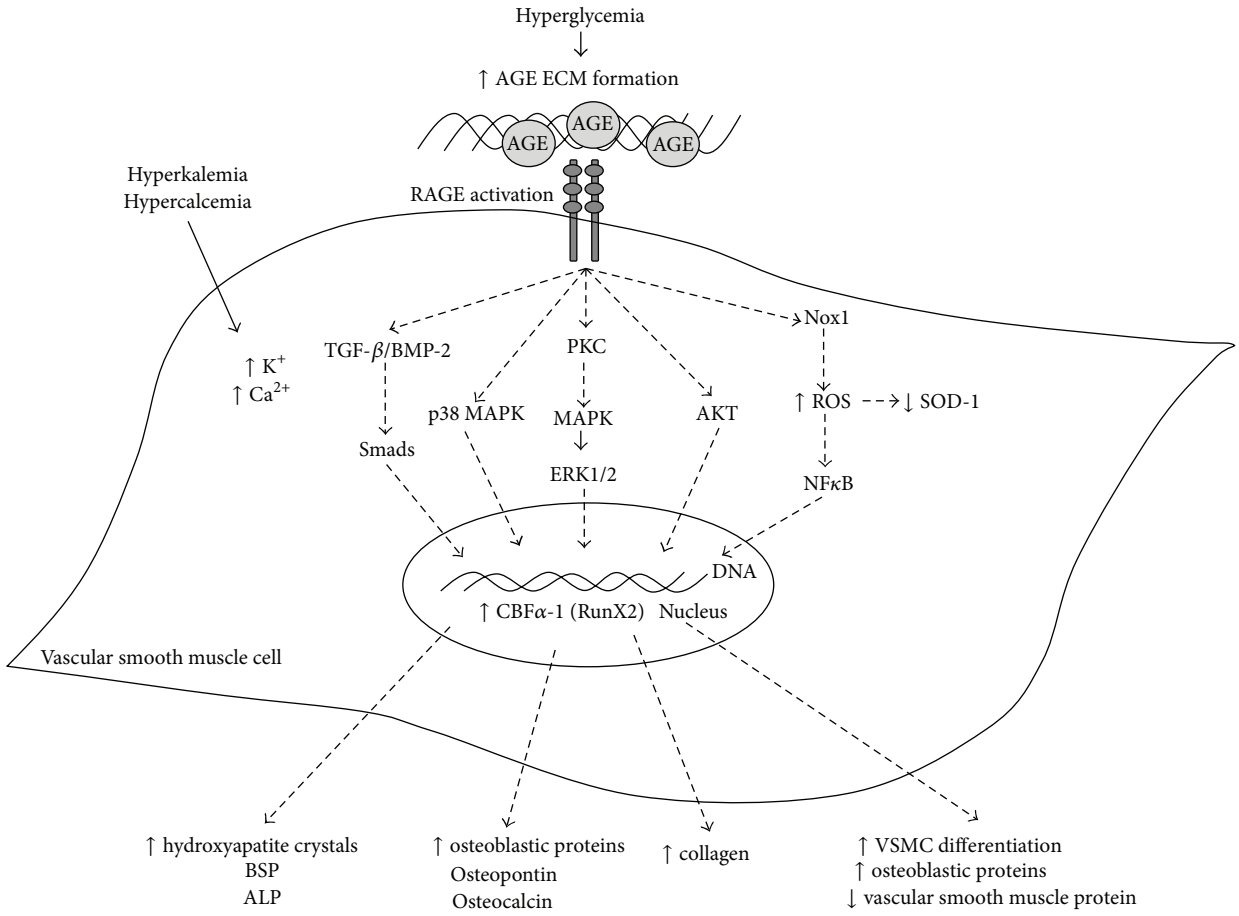

Figure 1: Schematic of AGE/RAGE signaling in diabetes-mediated vascular calcification.

Overall, the researchers demonstrated that RAGE signaling interferes with the expression of smooth muscle phenotype markers in A7r5 cells. The loss of smooth muscle phenotype markers offered an explanation for the changes in smooth muscle mechanical cell properties as AGE/RAGE signaling increased. There was also an increased granularity within the A7r5 cells demonstrating a visual change in cell morphology due to increased RAGE signaling. While the overall actin density was unchanged with AGE-HAS treated cells, Young's modulus, a measure of elasticity, revealed that basal cell rigidity was significantly increased indicating a stiffer, less elastic cell type. Protein expression levels of phosphorylated myosin light chain (MLC) were also measured to determine changes in contractile function and actin-myosin-mediated motor activity. These results revealed that no changes in contractile function occurred when A7r5 cells were treated with AGE-HSA. Taken together, increased AGE/RAGE signaling alters the mechanical properties of VSMCs resulting in a stiffer, less compliant cell type.

6. Conclusion

AGE/RAGE signaling is a complex and intricate cascade and has been studied in many different disease states. Particularly, diabetes-mediated vascular calcification exhibits several factors that allows for AGE/RAGE signaling to heavily influence both cellular and systemic responses. Vascular calcification has been demonstrated to increase bone matrix proteins through PKC signaling in hyperglycemic and calcification conditions. AGEs-induced vascular calcification caused downregulation of VSMCs markers and an upregulation of bone matrix proteins, thus, suggesting that the VSMCs undergo a phenotypic switch to an osteoblast-like cell. RAGE signaling can also mediate VSMC calcification through a number of mitogenic pathways. Of those, the p38 MAPK pathway was demonstrated to be an essential component for AGE/RAGE mediated VSMC differentiation. Fetuin A was also shown to play a more controversial role in vascular calcification. Fetuin A acts as a mediator for both procalcification by artificially selecting for AGEs as a RAGE ligand as well as anticalcification in certain models of CDK. Fetuin-A represents an exciting area for more work to be done to understand its role in vascular calcification as a diabetic complication. AGE/RAGE signaling has been implicated in oxidative stress associated with diabetes-mediated vascular calcification through activation of Nox-1, TGF-β mediated fibrosis, NFκB, and ERK1/2 pathways and decreased expression of SOD-1. Researchers found that pharmacological

agents and certain antioxidants decreased the level of calcium deposition in AGEs-induced diabetes-mediated vascular calcification. Overall, the role of AGE/RAGE signaling in diabetes-mediated vascular calcification was attributed to oxidative stress and the phenotypic switch of VSMCs in AGEs-induced calcification conditions as shown in Figure 1. Future direction in understanding vascular calcification as a diabetic complication could include utilizing RAGE knockout mice to examine the effects of systemic inhibition of RAGE on diabetes-mediated vascular calcification. Also, the role of fetuin-A could be better examined to understand the interplay of this biomarker and AGE/RAGE signaling in type II diabetes.

Disclosure

Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

All authors contributed equally to this paper.

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Review Article

Oxidative Stress in Diabetic Nephropathy with Early Chronic Kidney Disease

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The increase in the prevalence of diabetes mellitus (DM) and the secondary kidney damage produces diabetic nephropathy (DN). Early nephropathy is defined as the presence of microalbuminuria (30–300 mg/day), including normal glomerular filtration rate (GFR) or a mildly decreased GFR (60–89 mL/min/1.73 m^2), with or without overt nephropathy. The earliest change caused by DN is hyperfiltration with proteinuria. The acceptable excretion rate of albumin in urine is <30 mg/day. Albuminuria represents the excretion of >300 mg/day. Chronic kidney disease (CKD) is characterized by abnormalities in renal function that persist for >3 months with health implications. Alterations in the redox state in DN are caused by the persistent state of hyperglycemia and the increase in advanced glycation end products (AGEs) with ability to affect the renin-angiotensin system and the transforming growth factor-beta (TGF-β), producing chronic inflammation and glomerular and tubular hypertrophy and favoring the appearance of oxidative stress. In DN imbalance between prooxidant/antioxidant processes exists with an increase in reactive oxygen species (ROS). The overproduction of ROS diminishes expression of the antioxidant enzymes (manganese superoxide dismutase, glutathione peroxidase, and catalase). The early detection of CKD secondary to DN and the timely identification of patients would permit decreasing its impact on health.

1. Introduction

Diabetes mellitus (DM) is a metabolic disturbance that is characterized by dysfunction in the secretion and response to insulin. The International Diabetes Federation (IDF) predicts that the prevalence of DM will increase from 285 million persons affected in 2010 to 439 million in 2030 (an increase of ~50%). In 2009, it was reported that diabetic nephropathy (DN) causes ~44% of all cases of chronic end-stage renal disease (ESRD) in the United States [1]. Diabetes mellitus contributes, in large part, to the high costs of health care and the increase in mortality from the increased incidence of DN that leads to ESRD, because patients must be inscribed in a program of renal replacement therapy (RRT), whether it be dialysis or kidney transplant [2]. In order for DN to occur several factors should coincide, among which are the effect of genetic susceptibility, hyperglycemia, activation of the polyol pathways, activation of the renin-angiotensin system, activation of the protein kinase C pathway, increase in the advanced glycation end products (AGEs), glomerular hyperfiltration, and the production of reactive oxygen species (ROS) [3].

The first histological changes in DN are considered to be detectable two years after the diagnosis of DM [4]. The DN remains asymptomatic until later stages when the intervention makes it impossible to detain progression of the illness. Therefore, it is urgently necessary to detect the illness before the appearance of complications secondary to DM [5].

2. Early Kidney Changes

Normal kidney function is characterized as a glomerular filtration rate (GFR) of ≥90 mL/min/1.73 m^2 without

Table 1: (a) Categories of glomerular filtration rate in chronic kidney disease. GFR categories mentioned are based on the agreed-upon modifications reported in [6]. (b) Categories of albuminuria in chronic kidney disease. Shown are the categories that correspond to the excretion rate of urinary albumin. All are complications caused by diabetes mellitus. Modified and reported in [6].

(a)

Category of glomerular filtration rate (GFR)		
Category	GFR (mL/min/1.73 m^2)	Term
G1	≥90	Normal or high
G2	60–89	Slightly decreased
G3a	45–59	Slight or moderately decreased
G3b	30–44	Moderately-to-severely decreased
G4	15–29	Severely decreased
G5	<15	Renal insufficiency

(b)

Category	AER (mg/24 h)	AER (mg/mmol)	AER (mg/g)	Term
A1	<30	<3	<30	Normal to slightly elevated
A2	30–300	3–30	30–300	Moderately elevated
A3	>300	>30	>300	Severely elevated

AER: albumin excretion rate.

albuminuria. The GFR can be evaluated using the equations of the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) or the formula of the Modification on Diet in Renal Disease (MDRD) Study [7, 8]. As such, early nephropathy is defined by the presence of microalbuminuria (30–300 mg/day), including a normal or mildly diminished GFR (60–89 mL/min/1.73 m^2), with or without manifested nephropathy [9].

Chronic kidney disease (CKD) is defined as abnormalities in the kidney structure or function for >3 months accompanied by structural damage as evidenced by histopathological studies or images, with health implications [8]. The ESRD is classified according to the guidelines of the initiative of results of quality in kidney disease (National Kidney Foundation, KDOQI Guidelines) [10]. The GFR is one of the most utilized markers for the diagnosis and follow-up of CKD and should be calculated in all patients with DM, based on evaluation in 5 categories of GFR function: G1, GFR ≥90 mL/min/1.73 m^2; G2, with 60–89 mL/min/1.73 m^2; G3, a stage that is divided into G3a with GFR from 45–59 mL/min/1.73 m^2 and G3b with 30–44 mL/min/1.73 m^2; G4, corresponding to 15–29 mL/min/1.73 m^2; and G5, known as ESRD established by <15 mL/min/1.73 m^2 [11] (Table 1(a)). The stages of G1 and G2 are considered early CKD; G3a and G3b are considered overt nephropathy [8, 9, 12], while G4 and G5 are considered advanced stages [13, 14].

The earliest changes caused by DN, even before alterations to the serum creatinine, are due to the hemodynamic changes of glomerular hypoperfusion with an initial increase in GFR known as hyperfiltration [9] which favors albumin leakage from the glomerular capillaries, causing the appearance of proteins in the urine [15]. In fact, the discovery of urinary albumin is the recommended method of detection because it manifests in the majority of nephropathies of chronic evolution and directly affects the sensitivity of the glomerular permeability [16]. Microalbuminuria corresponds to alterations in the excretion of albumin secondary to changes that occur in the glomerulus [17].

3. Albuminuria

Albuminuria (including the detection of very low levels) and the albumin-creatinine ratio (A/Cr of 3.5 mg/g) are both considered established risk factors for the progression of cardiovascular disease (CVD) (cerebrovascular accident or acute myocardial infarction) [8]. In healthy people, the acceptable excretion rate of albumin in urine is <30 mg/day, a figure that corresponds to the albuminuria stage A1 [11]. In current clinical practice, it is recommended to consider the stage as A2 when there is a moderate increase of microalbuminuria [11]. The lowest variant of albuminuria in A2 stage is defined as the albumin excretion rate of 20 mcg/min, which is equivalent to 30 mg/day or 30 mg/g of the A/Cr. The highest variant considered in A2 stage is the excretion of 200 mcg/min, equivalent to 300 mg/day and A/Cr of 300 mg/g. Consequently, the microalbuminuria range between 30 and 300 mg/day must be considered for stage A2, as well as in the 24 h collection of urine or the A/Cr. It is estimated that A2 stage progresses to stage A3 ~ 2-3% per year and is associated with a decrease in GFR. It is important to consider A2 stage as a risk factor for progression of the illness to stage A3, although not all patients progress and some even return to the A1 stage. However, it is fundamental to monitor levels of albuminuria since DN can progress relatively quickly to CKD [10]. If the albuminuria is >300 mg/day or the A/Cr is 300 mg/g, it is classified as stage A3 and represents a severe increase in the excretion of urinary albumin [18] (Table 1(b)).

The prevalence of microalbuminuria in the Mexican population with DM is about 40–50%, including all of the transient causes. However, if you exclude those, the persistence of microalbuminuria in the Mexican population is positive in a relatively small sample [9, 19]. It should

also be mentioned that macroalbuminuria in the Hispanic population with DM is similar to that reported in the non-Hispanic population [20].

There are some tests available which are relatively low in cost for the early diagnosis of moderate albuminuria. The 24 h urine collection is widely considered the gold standard in diagnostic testing. This test should be confirmed by two additional samples collected over the course of 3–6 months, since the positive diagnosis of stage A2 is determined by 2-3 abnormal tests. It should also be noted that the 24 h urine collection can be tricky and is, above all, susceptible to errors and should not be used when conditions exist which might increase the urinary excretion of albumin, such as in urinary tract infections, or when there is hematuria and other kidney alterations [21]. However, the urinary proteinuria/urinary creatinine ratio, defined as the concentration of proteins in urine divided by the creatinine concentration in a randomly collected urine sample, has been proposed as an alternative to the 24 h urine collection [22], with adequate correlation to the latter test [23].

4. Diabetic Nephropathy

Diabetic nephropathy is characterized by albuminuria (>300 mg/day) and a reduced GFR [24]. It should be considered that the albuminuria is sometimes present at the moment when DM is diagnosed, after the kidney has been exposed to chronic hyperglycemia since the prediabetic phase. The mechanisms implicated in the pathogenesis of DN are multiple and complex. The first hemodynamic changes of glomerular hypoperfusion and hyperfiltration favor the leakage of albumin from the glomerular capillaries. Also, structural changes are produced as being characterized by thickening of the glomerular basement membrane, glomerulosclerosis, and expansion of the mesangial cells that lead to kidney fibrosis [25]. Even when the clinical manifestations of DN include diminished GFR and increased levels of urinary excretion of albumin, a substantial proportion of patients with DM have low GFR without albuminuria [26]. Fortunately, only one-third of patients with DM develop nephropathy [27]. However, poor glucose control, arterial hypertension, the increase in cholesterol, and the activation of mediators of inflammation and oxidative stress favor the progress of nephropathy to advanced stages [11]. In fact, recent studies suggest that patients with DM can begin to show signs of kidney disease before the diagnosis of albuminuria [28].

5. Triggers of Diabetic Nephropathy

5.1. Hyperglycemia. Hyperglycemia seems to be the driving force for the progressive destruction of the glomeruli. The chronically elevated levels of blood glucose lead to the formation of AGEs with posterior hyperfiltration (potential GFR increase of 5–10%) causing glomerular hypertrophy [29]. Hyperglycemia also produces mechanical tension and frictional force which occur in tandem as a result of the hemodynamic changes to the glomeruli, which leads to the liberation of numerous cytokines, proinflammatory markers, and growth factors which stimulate various pathways of oxidative stress [10]. When the DN progresses to later stages, there will be a frank decrease in GFR [30]. Therefore, DN remains substantially underdiagnosed and insufficiently treated [15]. The glycated hemoglobin A1c (HbA1c) was added to the standards of control by the American Diabetes Association (ADA) as a marker of the presence or severity of hyperglycemia in DM, since it exhibits less biological variability than glucose levels and it responds effectively to diet and treatment [31].

6. Genetic Factors in Diabetic Nephropathy

Family histories of nephropathy without hypertension are more frequent in patients with nephropathy than in patients with normal function. This suggests the important role of genetic and family factors involved in the development of nephropathy in some patients with type 2 DM [30].

7. Arterial Hypertension

Management of arterial pressure is fundamental in patients with CVD to slow the progression of atherosclerotic changes [23]. Diabetes mellitus increases the risk of CVD 2-3 times with the increase in systolic arterial pressure [32]. Arterial hypertension seems to play an important role in DN, since the higher the systolic arterial pressure and the longer the duration of the hypertension, the more serious the nephropathy. It is recommended to control the blood pressure in ranges <140/90 mmHg in all patients with DM, although it is known that 30–50% of patients with DM concomitantly suffer from arterial hypertension [33]. Until recently, the ADA guidelines focused on the objective of achieving systolic arterial pressure of <130 mmHg and diastolic arterial pressure of <80 mmHg in order to decrease proteinuria and slow progression of the DN [10]. The inhibitors of the angiotensin converting enzyme (ACE) or the angiotensin II receptor blockers (ARB) are considered the first line in the management and prevention of the appearance of albuminuria. The use of ACE inhibitors and ARBs in the management of DN seems to prevent progression of the CKD [12]. The ACE inhibitors, like the ARBs, reduce arterial pressure, decreasing the vasoconstrictor effect of the angiotensin II and reducing fibrous infiltration and inflammatory processes in the glomeruli, blocking other secondary signaling pathways. However, the dual therapy of both medications is not recommended in the same patient [34]. It is worth mentioning that the arterial pressure seems to be reasonably well controlled in patients who attend centers of primary medical care [20]. Among the medications initially used by the family doctor is Captopril. As an antecedent, this medication was initially studied in insulin dependent patients. Its administration resulted in a 50% reduction in final results of death, dialysis, and transplant, with the additional benefit of protecting the glomerular filtration [35]. Losartan has been studied in patients with type 2 DM, since it favors a reduction in the incidence of increase in creatinine and the decrease in proteinuria in 35% compared to the conventional antihypertensive treatment [36]. Telmisartan and Enalapril are equivalent in offering significant kidney protection [37].

8. Oxidative Stress in Diabetic Nephropathy

Oxidative stress results from the link with the majority of molecular events that underline the pathological process in DN. It is related to alterations in the redox state caused by the persistent hyperglycemic state and the increase in AGEs. These events affect the renin-angiotensin system and the signaling of the transforming growth factor-beta (TGF-β), producing chronic inflammation and glomerular and tubular hypertrophy. The renal fibrosis is due primarily to the accumulation of the mesangial cells, favoring the depositing of extracellular matrix (ECM), the thickening of the tubular and glomerular membranes, the dysfunction of podocytes, and the appearance of apoptosis. All of these events are redox alterations that lead to the appearance of albuminuria, proteinuria, glomerulosclerosis, and tubule-interstitial fibrosis [38]. When the redox equilibrium is slightly altered, be it through prolonged increase in the production of ROS or through inefficient antioxidant mechanisms, it can give way to pathological processes. The slight increase in ROS above the physiological limit can induce significant conformational changes in the lipids, proteins, carbohydrates, and nucleic acids, which leads to distorted interactions of the cellular functions. Oxidative stress in DN has the ability to act as a trigger, modulator, and link within the complex web of pathological events that occur in DN. There are various molecular events that underlie and connect the metabolism, inflammation, and the oxidation in DN. It is known that ROS are molecules that can be beneficial or harmful to the vital processes of redox-sensitive signaling, since the redox state can propagate and adjust the signals from the cellular membrane to the nucleus [39]. Oxidative stress and changes in the AGEs reflect the metabolic and oxidative behaviors that are produced by DM which interfere in multiple signaling pathways [40].

The accumulation of AGEs can be considered as a useful noninvasive marker for tissue damage in DM. A new AGE similar to the 3-deoxyglucosone, methylglyoxal, methionine sulfoxide, and the 2-aminoadipic acid has recently demonstrated having some prognostic power with respect to the progression of DN. However, due to the sophisticated methods required for its detection, it is still far from use in practice. In DN it is frequent to find the conventional markers of oxidative stress in serum, urine, and various organs, among which are the products of lipid peroxidation (4-hydroxynonenal, malondialdehyde) and protein carbonyl groups [32]. There are few valid markers of oxidative stress in the diagnosis and early prognosis of DN. Therefore, deciphering the molecular bases of oxidative stress in DN and other pathologies is required [33]. In an oxidative environment, the polyol pathways promote the generation of AGEs such as N-carboxymethyl-lysine and pentosidine and organize the changes that lead to complications from DM [41]. The AGEs propagate metabolic signals through the interaction of various specific receptors known as RAGEs (the macrophage scavenger receptor and the galectin-3) on inducing the proliferation, apoptosis, autophagy, or migration of the cells, depending on the target cell [42]. The intracellular production of ROS is incited by the AGE-RAGEs interaction [43] through the activation of the peroxisomes proliferator gamma receptor [44]. The transcription factors NFk-B, AP-1, and SP-1 are even further activated by the signaling of the redox-sensitive pathways, which provokes a large quantity of proinflammatory and profibrotic responses. The AGEs and RAGEs are also capable of inducing themselves with the increase in expression of RAGEs, thus augmenting kidney failure. Another factor to consider is age because an increase in age activates cascades, which suggests that the prevention and treatment of DN should be centered not only in the early control of glycemia but also in the limitation of factors related to oxidative stress and the formation of AGEs [45].

9. Oxidative Stress in CKD

It is demonstrated that the main cause of morbidity and mortality in patients with CKD is due to CVD and that the oxidative stress together with the subclinical inflammatory state is ultimately responsible for the generation of atherosclerotic plaque [46], since the relationship between oxidative stress and CVD in RRT, with an emphasis on the effects of the loss of antioxidant substances through the filter membranes in hemodialysis or the distinct solutions of peritoneal dialysis, has been demonstrated [47]. In advanced CKD, without exposing the patient to the distinct influences of RRT, oxidation has been demonstrated in patients by what have been called the products of peroxidation: oxidation-reduction glutathione, nuclear and mitochondrial 8-oxo-deoxyguanosine, superoxide dismutase (SOD), glutathione reductase, glutathione peroxidase (GPx), catalase, F2 isoprostanes, and carbonyl proteins. In the present study, all of the markers had significant differences when compared to the control group; the 8-oxo-deoxyguanosine molecule of the nuclear DNA behaved like the most ideal marker to determine oxidative DNA damage. The authors concluded that elevated oxidative stress exists in patients with advanced CKD, which is probably established right from the early stages of the CKD [13].

10. Oxidative Stress in Early CKD

Limited information exists on oxidative stress in early CKD [48, 49]; however, the generation of NAPDH oxidase-dependent ROS has been reported as abnormally elevated in patients with CKD in G1 and G2 stages [50]. In these same stages, a decrease in the activity of SOD as well as the GPx, compared to controls, has been reported. Also, the activity of some antioxidants had a negative correlation to the levels of asymmetric dimethylarginine but positive correlation with endothelium-dependent vasodilation of the brachial artery [51].

11. Antioxidants

The local and systemic oxidative stress that underlies the pathological characteristics of DN is the result of the imbalance within the production of oxidants/antioxidants,

since the oxidative aggression is balanced through powerful antioxidant mechanisms. In uremic patients, there is imbalance with an increase in ROS. The overproduction of ROS induced by hyperglycemia decreases the expression of the manganese superoxide dismutase (mSOD) through the PI3K-AKT-FOXO3 pathway. The mSOD is considered the guardian of the generation of SOD in the mitochondria. The ROS have the ability to produce a decrease in the Sirtuins through modulation of the acetylation of the mitochondrial respiratory chain on stimulating the mitochondrial SOD [52]. Because the ROS are implicated in cellular signaling [53], they are capable of activating the proinflammatory processes and mitogenic cellular pathways that favor the progressive deterioration of kidney function with the appearance of kidney fibrosis and deterioration of the GPx [54], the SOD, catalase, and the nitric oxide synthases (NOS), all of which are considered the most important antioxidant enzymes which detoxify ROS in the kidney [55]. The SOD is the principle physiological defense against oxidative stress which reacts with the superoxide (O_2^-) to generate hydrogen peroxide (H_2O_2), which is degraded by the catalase and the GPx [56, 57]. The SOD has been demonstrated to suppress albuminuria, levels of TGF-β, collagen synthesis, and oxidative stress in experimental models [58]. The GPx uses glutathione to reduce H_2O_2 to peroxides of water, acting in conjunction with the peroxynitrite reductase [59].

12. Recommended Antioxidants

An extensive review has centered on the study of Curcumin (diferuloylmethane), a component of *Curcuma longa*; in several experimental studies, Curcumin has been demonstrated to modulate multiple molecules of cellular signaling such as the proinflammatory cytokines, apoptosis proteins, transcription factors, TGF-β, and diverse endogenous antioxidants [60]. A clinical study performed in 20 patients with type 2 DM and DN demonstrated that short-term supplementation with Curcumin attenuated the proteinuria and the expression of TGF-β and interleukin 8. It is important to mention that although Curcumin can be administered as adjuvant therapy, long-term studies with higher numbers of patients are required to confirm the results [61]. In the meta-analysis done by Bjelakovic et al., in 68 randomized trials that included more than 200,000 adults, they compared beta-carotenes, vitamins A, C, and E, and Selenium versus placebo or no intervention. This systematic review showed that only Selenium had the tendency to reduce all of the causes of mortality and that vitamins A and E augmented it, while only vitamin C did not demonstrate any effects [62]. Biesalski et al. reviewed the analysis done by Bjelakovic et al. and concluded that the dietary supplements for the prevention or treatment of chronic illnesses are more effective in patients who ingest insufficient doses, since apparently there is a threshold above which the ingestion of additional nutrients does not offer additional benefits. The threshold for ingestion of nutrients depends on age, gender, health status, and genetic polymorphisms [63].

13. Preventive Measures for DN

The early detection and treatment of DN include annual monitoring or more frequently if considered necessary depending on the levels of serum albumin, albuminuria, creatinine in the urine, and the GFR and the improvement and control of glycemia with the objective of achieving a HbA1c < 7% and initiation of ACE inhibitors or ARBs as a first line in managing the illness. It is recommended to initiate therapy with statins in patients <50 years old with concomitant ESRD and DM regardless of the coexistence with DM.

It is imperative to consider that the ESRD will continue augmenting and it is probable that health care systems are not capable of facing the costs. Therefore, the importance of prevention (primary and secondary) to stop the progression of kidney disease and the consequences of ESRD and diminish the associated causes that increase mortality like CVD needs to be emphasized. It is essential to detect patients with ESRD at the onset of clinical or laboratory signs in order to optimize their care and attention [6, 63, 64].

In conclusion, with the early detection of CKD secondary to DN, and with the adequate identification of patients, its impact on health could be diminished and thus, as a result, its impact on society.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Research Article

The Impact of Type 2 Diabetes on the Efficacy of ADP Receptor Blockers in Patients with Acute ST Elevation Myocardial Infarction: A Pilot Prospective Study

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Background. The aim of this study was to validate the impact of type 2 diabetes (T2D) on the platelet reactivity in patients with acute ST elevation myocardial infarction (STEMI) treated with adenosine diphosphate (ADP) receptor blockers. *Methods*. A pilot prospective study was performed. Totally 67 patients were enrolled. 21 patients had T2D. Among all study population, 33 patients received clopidogrel and 34 patients received prasugrel. The efficacy of ADP receptor blocker therapy had been tested in two time intervals using light transmission aggregometry with specific inducer and vasodilator-stimulated phosphoprotein phosphorylation (VASP-P) flow cytometry assay. *Results*. There were no significant differences in platelet aggregability among T2D and nondiabetic (ND) group. The platelet reactivity index of VASP-P did not differ significantly between T2D and ND group (59.4 ± 30.9% versus 60.0 ± 25.2% and 33.9 ± 25.3% versus 38.6 ± 29.3% in second testing). The number of ADP receptor blocker nonresponders did not differ significantly between T2D and ND patients. The time interval from ADP receptor blocker loading dosing to the blood sampling was similar in T2D and ND patients in both examinations. *Conclusion*. This prospective study did not confirm the higher platelet reactivity and higher prevalence of ADP receptor blocker nonresponders in T2D acute STEMI patients.

1. Background

Type 2 diabetes (T2D) is a strong and independent risk factor of cardiovascular disease in both men and women [1–3]. Diabetes not only increases the risk of acute coronary syndrome (ACS) but also increases the mortality associated with this acute event. T2D worsens the course of ACS and increases the risk of its complications including cardiogenic shock and death [4] and remains an independent predictor of worse prognosis of ACS, even after excluding the impact of older age, more severe coronary artery disease, and worse left ventricular function in patients with T2D [5]. Moreover, T2D is connected with platelet dysfunction [6, 7], which increases the risk of arterial thrombosis. Several recently published studies and case reports pointed at a failure in antiplatelet response to clopidogrel (the most frequently used ADP receptor blocker in ACS patients) which might be connected with insulin resistance and T2D. High clopidogrel on-treatment platelet reactivity is associated with increased risk of thrombotic adverse events including stent thrombosis [8–10]. The aim of this preliminary prospective study was to validate the impact of T2D in relation to the on-treatment

platelet reactivity in patients with acute ST elevation myocardial infarction (STEMI) treated with ADP receptor blocker therapy.

2. Methods

2.1. Study Design and Patients. A single centre, pilot prospective observational study in patients with acute STEMI was performed. All patients underwent coronary angiography and primary percutaneous coronary intervention (pPCI) of culprit coronary lesion. Totally 67 consecutive presentations of patients (37 men and 30 women; mean age 67 years; the youngest patient was 34-year-old and the oldest patient was 89-year-old) with acute STEMI and pPCI of coronary lesion were enrolled in this study. Patients with multivessel coronary disease planned for surgical revascularization, patients treated only conservatively, and hemodynamically unstable patients (i.e., patients in Killip class IV) had been excluded from this study. Additionally, patients with hypertensive crisis, kidney, and liver failure had been also excluded from study population. Moreover, patients with medication which could interfere with the action of ADP receptor blockers, such as omeprazole, fluconazole, or morphine were also excluded from this study. All patients should be ADPRB naïve prior to myocardial infarction. Patients with known and correctly diagnosed history of T2D had been assigned to T2D group. This group included 21 patients. In all patients without previous history of T2D the glycated hemoglobin (HbA1C) levels were assessed in order to exclude patients with undiagnosed T2D. Patients with HbA1C levels > 5.7% DCCT were considered to be patients with possibly undiagnosed T2D and these patients had been excluded from the study. Subsequently, a standard oral glucose tolerance test (75 g of glucose was administrated in 100 mL of water and a venous blood sample was taken two hours after the glucose administration) was performed in the rest of the patients without previous history of T2D one month after the hospital discharge. Patients with blood glucose value > 7.8 mmol/L two hours after the glucose administration had been also excluded from the study. Patients without previous history of T2D, with HbA1C levels < 5.7% DCCT and blood glucose value < 7.8 mmol/L shown in oral glucose tolerance test had been assigned to nondiabetic (ND) group. The basic demographic data and concomitant medication in the study population are shown in Table 1. Among all study population, 33 patients received clopidogrel (a loading dose of 600 mg followed by a maintenance dose of 75 mg/daily) and 34 patients received prasugrel (a loading dose of 60 mg followed by a maintenance dose of 10 mg/daily). Totally 22 patients enrolled in this study were >75 years old (9 T2D patients and 13 ND patients); all of these patients were clopidogrel-treated. All patients received aspirin therapy (a loading dose of 200–400 mg followed by a maintenance dose of 100 mg/daily) and a weight-adjusted unfractionated heparin therapy (100 IU/kg intravenously) prior to pPCI in order to prevent periprocedural thrombosis. No other antiplatelet or anticoagulant therapy was administrated in studied population. The drug compliance had been carefully monitored with a healthcare professional, who supervised all antiplatelet drug administration. Venous blood samples had been taken after obtaining the written informed content in all patients enrolled in this study in order to assess the on-treatment platelet reactivity using selected platelet function tests.

2.2. Platelet Function Testing. Blood samples had been taken using 3.8% citrate vacutainer blood collection tubes. Blood samples had been immediately analyzed within first 2 hours from blood sampling. The samples had been taken in the following time intervals:

Sample 1, at the time of patient arrival to cath laboratory: this sample aimed to test the efficacy of ADP receptor blocker given in loading doses prior to the urgent coronary angiography and pPCI of coronary lesion.

Sample 2, one hour after the administration of first ADP receptor blocker maintenance dose: this sample aimed to test the efficacy of in-hospital ADP receptor blocker therapy given in maintenance dosage.

The platelet reactivity was tested using light transmission aggregometry (LTA) with specific inducer (ADP) and vasodilator-stimulated phosphoprotein phosphorylation (VASP-P) flow cytometry assay.

Light Transmission Aggregometry (LTA). this method represents recently the "golden standard" of platelet function testing. ADP (10 μmol/L) was used as specific inducer for ADP receptor blocker efficacy testing. LTA was examined with Chrono-Log model 700 (Chronolog Corporation, Havertown, PA, USA). The platelet aggregability was assessed on the basis of the change in the plasma turbidity after the addition of the specific inducer. Residual platelet aggregability > 50% after the addition of ADP was considered to be high on-treatment platelet reactivity (HTPR).

VASP-P Flow Cytometry Assay. In this analysis, we used PLT VASP/P2Y12 assay kits (Diagnostica Stago, France). Sample of citrate blood was incubated with prostaglandin E1 (PGE1) and PGE1 + ADP (activated platelets). After cellular permeabilization by nonionic detergent, VASP-P is labeled by indirect no-wash immunofluorescence using a specific monoclonal antibody. Dual color flow cytometry analysis then allowed comparison of the 2 tested conditions. Analysis was carried out on FACSCalibur flow cytometer (BD Biosciences, San Jose, California). In the final step, the platelet reactivity index (PRI) was calculated using corrected mean VASP fluorescence intensities (MFIc) in the presence of PGE1 alone (resting platelets) or PGE1 + ADP simultaneously (activated platelets). Index represented the ratio of activated/resting platelets and was calculated according to the following equation:

$$\mathrm{PRI} = \frac{\mathrm{MFIc}^{\mathrm{PGE1}} - \mathrm{MFIc}^{[\mathrm{PGE1+ADP}]}}{\mathrm{MFIc}^{\mathrm{PGE1}}} \times 100. \quad (1)$$

The resulting value described PRI to ADP treatment in the range of 0% to 100%. Values of PRI above 50% were considered as determinant of HTPR and inadequate response to treatment.

Table 1: Demographic data and concomitant medication in studied acute STEMI patients.

	T2D group	ND group	Significance
Number of patients (men/women)	21 (11/10)	46 (26/20)	NS
T2D duration (years)	11.4 ± 7.6	—	N/A
HbA1C levels	7.2 ± 1.9%	4.03 ± 0.7%	$p < 0.001$
Age	72 (52–89)	65 (34–88)	NS
Body mass index (kg/m^2)	29.6 ± 4.2	28.6 ± 4.2	NS
Beta blockers (% of patients/number of patients)	90%/19 patients	89%/41 patients	NS
ACE inhibitors or AT1 blockers (% of patients/number of patients)	85%/18 patients	67%/31 patients	NS
Statins (% of patients/number of patients)	100%/21 patients	96%/44 patients	NS
Diuretics (% of patients/number of patients)	52%/11 patients	31%/14 patients	NS
Clopidogrel (% of patients/number of patients)	52%/11 patients	48%/22 patients	NS
Prasugrel (% of patients/number of patients)	48%/10 patients	52%/24 patients	NS

Table 2: On-treatment platelet reactivity and prevalence of ADP receptor blocker nonresponders in T2D and ND patients.

On-treatment platelet reactivity	T2D patients	ND patients	Significance
LTA with ADP induction (%)			
Sample 1	57.2 ± 26.4	50.3 ± 22.3	NS
Sample 2	45.9 ± 31.3	37.0 ± 20.8	NS
PRI of VASP phosphorylation (%)			
Sample 1	59.4 ± 30.9	60.0 ± 25.2	NS
Sample 2	33.9 ± 25.3	38.6 ± 29.3	NS
ADP receptor blocker nonresponders (% of patients/number of patients)	T2D patients	ND patients	Significance
ADP receptor blocker nonresponders			
Sample 1	52.4%/11 patients	56.5%/26 patients	NS
Sample 2	33.3%/7 patients	30.4%/14 patients	NS

2.3. Statistical Analysis. Data were in the first step checked for normality with the Shapiro-Wilk test. Normally distributed continuous or interval-scaled variables are presented as mean ± standard deviation (SD); otherwise median (M) and quartile ranges from the lower quartile to the upper quartile were used. Group effects (i.e., differences between T2D and ND groups) were tested with t-test in the case of normally distributed data or with Mann-Whitney U test when data distribution was asymmetrical. Differences between proportions (e.g., number of patients in T2D and ND groups) were tested with binominal tests. Categorical variables grouped in 2-way contingency tables were analyzed using chi-square tests. The significance of $p < 0.05$ was considered as a criterion for comparison between data sets with equal and unequal variances. The statistical analysis was performed with Statistica v. 7.0 (StatSoft Inc., Dell Software, Tulsa, Oklahoma, USA). Sample size calculation was based on the assumption of the incidence of HTPR among ADP receptor blockers-treated T2D patients reported in previously published studies [9, 10]. The primary aim of this study was to clarify possible differences in ADP receptor blockers on-treatment platelet reactivity according to T2D status. Choosing a two-sided value of 0.05, we estimated that an overall sample size of 20 T2D patients and 20 control (nondiabetic) patients would be sufficient for statistical analysis. To reach even more valid sample, we decided to enroll more than 20 patients in each of the compared groups (however, we were able to enroll only one more T2D patient who met the inclusion criteria of the study). The statistical analysis was consulted with a professional and designed prior to patient enrollment to achieve a valid statistical evaluation of the results of the study.

3. Results

The time interval from ADP receptor blocker loading dose administration to the collection of sample 1 was 1.8 ± 0.9 hours and to the collection of sample 2 was 20.5 ± 2.1 hours. The mean platelet aggregability after the induction with ADP was 52.5 ± 23.6% in sample 1 and 39.7 ± 24.5% in sample 2. Examination of VASP phosphorylation showed mean PRI 59.7 ± 26.9% in sample 1 and mean PRI 37.0 ± 27.8% in sample 2, respectively.

When comparing the T2D and ND group (Table 2) there were no significant differences in platelet aggregability after ADP neither in sample 1 nor in sample 2 (sample 1: 57.2 ± 26.4% versus 50.3 ± 22.3%, NS; sample 2: 45.9 ± 31.3% versus 37.0 ± 20.8%, NS). The PRI of VASP-P showed similar results: there were no significant differences between T2D and ND group neither in sample 1 (59.4 ± 30.9% versus 60.0 ± 25.2%, NS) nor in sample 2 (33.9 ± 25.3% versus 38.6 ± 29.3%, NS). The time interval from ADP receptor blocker loading dose administration to the sample collection was similar in T2D

Table 3: Platelet aggregability and PRI of VASP-P in prasugrel- and clopidogrel-treated patients with acute STEMI (analysis of combined T2D and ND patients and analysis of T2D patients, marked as T2D patients).

LTA with ADP induction (%)	Prasugrel-treated patients	Clopidogrel-treated patients	Significance
Sample 1	45.9 ± 26.2%	59.2 ± 18.8%	$p < 0.05$
Sample 2	24.9 ± 17.7%	51.7 ± 22.8%	$p < 0.001$
LTA with ADP induction (%) T2D patients	Prasugrel-treated T2D patients	Clopidogrel-treated T2D patients	Significance
Sample 1	43.3 ± 20.6	63.5 ± 22.8	$p < 0.05$
Sample 2	22.9 ± 16.9	60.3 ± 26.6	$p < 0.01$
PRI of VASP-P analysis (%)	Prasugrel-treated patients	Clopidogrel-treated patients	Significance
Sample 1	52.7 ± 29.7%	68.9 ± 19.9	$p < 0.05$
Sample 2	26.3 ± 24.3%	51.8 ± 22.8%	$p < 0.01$
PRI of VASP-P analysis (%) T2D patients	Prasugrel-treated T2D patients	Clopidogrel-treated T2D patients	Significance
Sample 1	46.8 ± 33.5	78.3 ± 13.0	$p < 0.05$
Sample 2	18.7 ± 15.7	56.8 ± 18.7	$p < 0.01$

and ND patients in both examinations (sample 1: 1.8 ± 0.9 hours versus 1.7 ± 0.9 hours; sample 2: 21.6 ± 2.2 hours versus 20.0 ± 1.9 hours).

Subsequently, an analysis of ADP receptor blocker nonresponders (Table 2) was performed. The difference in the prevalence of ADP receptor blocker nonresponders in T2D and ND patients did not reach statistical significance. However, the number of ADP receptor blocker nonresponders tended to be higher in T2D patients. In T2D patients an incomplete response on ADP receptor blocker (PRI > 50%) was identified in 52.4% of patients in first sample and in 33.3% in second sample. While in ND group, 56.5% of patients were nonresponders in first sample and 30.4% of patients did not respond sufficiently in second sample. No significant differences in the prevalence of ADP blocker nonresponders between T2D prasugrel-treated and ND prasugrel-treated patients were found (sample 1: 40.0% versus 41.7%, NS; sample 2: 10.0% versus 12.5%, NS). In addition, the prevalence of ADP receptor blocker nonresponders did not differ significantly in clopidogrel-treated T2D patients compared to clopidogrel-treated ND patients (sample 1: 63.6% versus 72.7%, NS; sample 2: 54.6 versus 50.0%, NS).

Prasugrel induced in this preliminary study significantly more potent platelet inhibition (Table 3) than that of clopidogrel. Prasugrel-treated patients had significantly lower residual platelet aggregation in both samples: 45.9 ± 26.2% versus 59.2 ± 18.8% ($p < 0.05$) in sample 1 and 24.9 ± 17.7% versus 51.7 ± 22.8% ($p < 0.001$) in sample 2, respectively. Similarly, the PRI of VASP-P was significantly lower in prasugrel-treated patients in sample 1, as well as in sample 2 (sample 1: 52.7 ± 29.7% versus 68.9 ± 19.9, $p < 0.05$; sample 2: 26.3 ± 24.3% versus 51.8 ± 22.8%, $p < 0.01$). Consistently, significantly lower residual platelet reactivity was found in prasugrel-treated T2D patients when compared to clopidogrel-treated T2D patients in both samples (sample 1: 43.3 ± 20.6% versus 63.5 ± 22.8%, $p < 0.05$; sample 2: 22.9 ± 16.9% versus 60.3 ± 26.6%, $p < 0.01$). Significantly better inhibition of ADP signaling pathway in prasugrel-treated T2D patients was demonstrated with significantly lower PRI of VASP-P in both samples (sample 1: 46.8 ± 33.5 versus 78.3 ± 13.0, $p < 0.05$; sample 2: 18.7 ± 15.7 versus 56.8 ± 18.7, $p < 0.01$).

4. Discussion

T2D is a strong and independent risk factor of acute STEMI. T2D increases the risk of future complications in acute STEMI patients. Endothelial dysfunction [11], abnormalities in coagulation and fibrinolysis [12], and platelet dysfunction [6, 7] are important factors of worsen prognosis of acute STEMI in T2D patients. However, several studies pointed at a failure in antiplatelet response to clopidogrel which might be connected with insulin resistance and T2D [8–10]. These studies consistently identified higher residual on-treatment platelet reactivity in clopidogrel-treated T2D patients and higher number of clopidogrel nonresponders among diabetic patients. Moreover, HTPR was in these studies consistently connected with higher risk of adverse ischemic events after PCI. The mechanism of this phenomenon remains unclear. The study performed by Erlinge et al. [9] showed that platelets of diabetic patients with clopidogrel HTPR responded well to ex vivo administration of clopidogrel active metabolite. This finding suggests a potential interaction between T2D and pharmacokinetic processes of clopidogrel metabolism.

On the other hand, the results of this preliminary prospective observation did not confirm the significantly higher residual platelet reactivity or significantly higher prevalence of HTPR in T2D acute STEMI patients undergoing pPCI of culprit coronary lesion. The exact explanation of this observation is recently missing. The time interval from drug administration to blood sample collection did not differ significantly in T2D and ND patients neither in sample 1 nor in sample 2. The differences in time interval from drug

dosing to blood sampling therefore cannot explain the results obtained in this study.

Another possible explanation may be the fact that the majority of studies pointing on the higher prevalence of HTPR among T2D patients were performed on a sample of stable coronary/ischemic heart disease patients. It is generally accepted that patients with acute STEMI represent a special group with different clinical and risk profile. It is therefore possible that results obtained from the studies on stable patients might not be fully applicable in high risk ACS patients. However, study performed by Cuisset et al. identified high prevalence of clopidogrel nonresponders (50% of patients) also in T2D patients undergoing PCI for ACS [13]. In the light of these data, the real influence of acute coronary syndrome itself on the prevalence of HTPR in T2D patients seems not to be probable.

An alternative explanation of similar on-treatment platelet reactivity in T2D and ND patients in this study might be a possibility that the failure in antiplatelet response is in T2D patients specifically associated with clopidogrel. The administration of newer ADP receptor blockers might not be connected with such a failure in on-treatment response [14]. In fact, prasugrel administration in the TRITON-TIMI 38 trial achieved the most pronounced clinical benefit just in T2D patients [15]. Prasugrel was in our preliminary study quite frequently used among T2D (48% of patients), as well as among ND patients (52% of patients). Prasugrel induced in our study significantly more potent platelet inhibition than that of clopidogrel in both samples. The efficacy of newer ADP receptor blockers among high risk T2D acute coronary syndrome patients was consistently proven in a recent single centre randomized clinical study performed by Laine et al. [16]. It is therefore possible that more frequent use of newer ADP receptor blockers (such as prasugrel) would resolve the insufficient platelet inhibition, which might be seen in clopidogrel-treated T2D patients. Nevertheless, the relationship between T2D and ADP receptor blocker on-treatment platelet reactivity remains inadequately explained [17–19] and further studies would be needed for the final clarification of this issue.

5. Limitations

There were some important limitations of our analysis. First, this study had a prospective observational design and not a randomized double blinded one. The decision on ADP receptor blocker therapy strategy (clopidogrel versus prasugrel) was left to the physician who performed the diagnostic ECG record (general practitioner, cardiologist, ED physician, etc.). Therefore the data obtained in this study do not have the evidence power of data from a randomized double blinded trial. Second, a low sample size might be a limitation of the study. A relatively small patient sample cannot guarantee significant power. Third, a relatively short time interval from ADPRB loading dosing to first sampling (especially in the settings of acute coronary syndrome) might be another limitation of this study. It is already known that time to peak platelet inhibition is generally prolonged in acute STEMI patients and also in stable coronary artery disease patients with T2D [19]. However, in this study, we wanted to test the impact of T2D on the on-treatment response in previously ADPRB naïve acute STEMI patients undergoing pPCI. Therefore we decided to test the antiplatelet response prior to pPCI and one day after the procedure rather than after a prolonged ADPRB administration. Nevertheless, this fact could affect the on-treatment platelet reactivity detected in our study. Finally, the drug compliance was not proven by a laboratory testing (measurement of clopidogrel metabolite, etc.). Although, the drug compliance was in this study carefully monitored by a healthcare professional who supervised all antiplatelet drug administrations, the exact confirmation of drug compliance with laboratory assessment is missing. The results of this study should be confirmed in large, similarly designed, randomized trial.

6. Conclusion

This prospective study did not confirm the higher residual platelet reactivity and higher prevalence of HTPR in T2D acute STEMI patients undergoing pPCI of culprit coronary lesion. However, the results of this study are preliminary and further studies on larger sample sizes would be definitely needed for the final clarification of this issue.

Abbreviations

T2D: Type 2 diabetes
STEMI: ST elevation myocardial infarction
ADP: Adenosine diphosphate
VASP-P: Vasodilator-stimulated phosphoprotein phosphorylation
pPCI: Primary percutaneous coronary intervention
ND: Nondiabetic
ACS: Acute coronary syndrome
HTPR: High on-treatment platelet reactivity
HbA1C: Hemoglobin A1C
DCCT: Diabetes Control and Complications Trial
LTA: Light transmission aggregometry
PGE1: Prostaglandin E1
MFIc: Corrected mean fluorescence intensity
PRI: Platelet reactivity index
SD: Standard deviation
ECG: Electrocardiography
ED: Emergency department.

Ethical Approval

This research was done according to ethical standards and was approved by the local ethical committee.

Consent

The patients agreed to participate in the research and signed written informed consent for study participation.

Competing Interests

The authors have no conflict of interests to declare.

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Clinical Study

Topical Administration of Pirfenidone Increases Healing of Chronic Diabetic Foot Ulcers: A Randomized Crossover Study

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Only 30 percent of chronic diabetic foot ulcers heal after 20 weeks of standard treatment. Pirfenidone is a drug with biological, anti-inflammatory, and antifibrotic effects. The aim of this study was to evaluate the effect of topical pirfenidone added to conventional treatment in noninfected chronic diabetic foot ulcers. This was a randomized crossover study. Group 1 received topical pirfenidone plus conventional treatment for 8 weeks; after this period, they were switched to receive conventional treatment only for 8 more weeks. In group 2, the order of the treatments was the opposite. The end points were complete ulcer healing and size reduction. Final data were obtained from 35 ulcers in 24 patients. Fifty-two percent of ulcers treated with pirfenidone healed before 8 weeks versus 14.3% treated with conventional treatment only ($P = 0.025$). Between 8 and 16 weeks, 30.8% ulcers that received pirfenidone healed versus 0% with conventional treatment ($P = 0.081$). By week 8, the reduction in ulcer size was 100% [73–100] with pirfenidone versus 57.5% with conventional treatment [28.9–74] ($P = 0.011$). By week 16, the reduction was 93% [42.7–100] with pirfenidone and 21.8% [8–77.5] with conventional treatment ($P = 0.050$). The addition of topical pirfenidone to conventional treatment significantly improves the healing of chronic diabetic noninfected foot ulcers.

1. Introduction

Type 2 diabetes (T2D) is a chronic disease with an increasing incidence worldwide. The majority of T2D costs are derived from its complications. Diabetic foot is one of the most common and devastating complications of diabetes. It remains the leading cause of nontraumatic lower-extremity amputation [1, 2]. The annual incidence of diabetic foot ulcers varies between 1.9% and 2.2%, with a prevalence of 7.5% to 12% [3]. The risk of amputation is associated with the presence of sensory peripheral neuropathy, peripheral vascular disease, Charcot joint, ulceration, and the presence of infection [4]. Fifty-six percent of diabetic foot ulcers will develop infection and 20% of them will end up in a lower-extremity amputation [5].

A number of chemical mediators are involved in the tissue repair process such as cytokines and growth factors. Within this complex process, the transforming growth factor beta (TGF-β) plays a key role regulating the development, differentiation, growth, and apoptosis of most cells [6]. As inflammation develops, there is an increase in TGF-β production by inflammatory cells. TGF-β in turn activates monocytes by increasing gene expression of proinflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α). Metalloproteinases (MMPs) are also important in the repair process of damaged tissues. The natural substrates

of MMPs include major proteins of the extracellular matrix (ECM) such as collagen, elastin, and proteoglycans [7].

There have been advances in the management of diabetic ulcers. Therapies including growth factors, bioengineered skin, tissue grafts, hyperbaric oxygen, negative pressure wound therapy, and other novel approaches to stimulating wound healing have demonstrated healing rates of around 40% in noninfected diabetic foot ulcers [8].

1-Phenyl-5-methyl-2-[1H]-pyridone (pirfenidone) is a synthetic chemical molecule that acts as a selective cytokine regulator, providing its action by anti-inflammatory and specific antifibrotic properties. Pirfenidone acts as a modulator of TNF-α, TGF-β, fibroblast growth factor (FGF), platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF), which are cytokines involved in the inflammatory-fibrotic process. This results in a reduced expression of TGF-β by a direct inhibition of furin, a proprotein convertase. These actions balance the production of MMPs [9]. These effects are associated with an improvement in reepithelialization, inflammation, and fibrosis.

Pirfenidone has shown utility in the treatment of patients with wounds, burns, and scars without serious adverse events [10]. Topical pirfenidone may improve healing of diabetic foot ulcers and could be an option as an adjuvant therapy. Therefore, the aim of this study is to evaluate the effect of pirfenidone added to conventional treatment on noninfected diabetic foot ulcers assessing the rate of complete wound closure and the change in ulcer size. In addition, safety of pirfenidone will be evaluated.

2. Material and Methods

2.1. Trial Design. This was a prospective controlled randomized crossover study. The protocol was approved by the Comite de Etica en Investigacion del Instituto Nacional de Ciencias Medicas y Nutricion. All subjects agreed to participate and provided informed consent before starting the study. The trial was registered under Clinical Trials NCT02222376.

2.2. Participants. Subjects who attended the Diabetic Foot Clinic at the Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran in Mexico City were recruited. Wagner grading system was used to classify the ulcers for inclusion. Grade 0 is a patient at risk or with a postulcerative diabetic foot without ulcer. Grade 1 is a full-thickness ulcer not involving tendon, capsule, or bone. Grade 2 is an ulcer that involves tendon or capsule, without abscess or osteomyelitis. Grade 3 is a deep ulcer with abscess or osteomyelitis. Grade 4 is an ulcer with gangrene in a portion of the forefoot. Finally, grade 5 is an ulcer with extensive gangrene [12].

The inclusion criteria were the following: men and women with T2D, being older than 18 years of age, being with a diabetic foot ulcer grade Wagner 1 or 2, ulcer size $\geq 1\,cm^2$, and being with duration of at least 8 weeks. The exclusion criteria included infected ulcers, presence of an ankle brachial index (ABI) <0.4, ulcers due to a different cause such as venous insufficiency, inability to attend to the weekly evaluations, use of systemic or topical diabetic foot ulcer treatments, immunosuppressant treatment, connective tissue diseases, pregnancy, and lactation. None of the patients received antibiotic treatment previously or during the study. The elimination criteria were an attendance to <75% of the evaluations, allergy to pirfenidone, development of ulcer infection, and occurrence of other serious diseases requiring hospitalization.

2.3. Interventions. Conventional treatment consisted of weekly ulcer cleansing with saline, debridement using a surgical blade, maintenance of a moist environment, and covering with sterile gauzes. In addition, patients were instructed to perform daily cleansing with saline-moistened gauzes and offloading the affected extremity. Topical pirfenidone treatment consisted of applying pirfenidone over the ulcer twice a day.

All patients completed a pretreatment phase of 7 days receiving conventional treatment. After this week, participants were randomly assigned to one of the two groups. Group 1 received conventional treatment in combination with topical pirfenidone for the first eight weeks and at the end of this period they were switched to conventional treatment only for the remaining 8 weeks. Group 2 received conventional treatment only for the first 8 weeks and at the end of this period they were switched to conventional treatment in combination with topical pirfenidone for another 8 weeks. Patients were evaluated weekly during the 17 weeks at the diabetic foot clinic.

Weight, height, and blood pressure were measured using standardized techniques. The body mass index was calculated as the weight in kilograms divided by the squared height in meters. In addition, all ulcers were categorized using the classification of the International Working Group on the Diabetic Foot (IWGDF), abbreviated with the acronym PEDIS, which stands for perfusion, extent (size), depth (tissue loss), infection, and sensation (neuropathy) [13]. To evaluate the presence of lower-extremity vascular disease, the ABI was calculated and classified as follows: normal from 0.9 to 1.3, peripheral artery disease (PAD) $\leq$ 0.9, and severe PAD < 0.4. When the ABI was > 1.3, it was considered as a noncompressible vessel [11]. After debridement, the length and width of the ulcer were measured with a standard ruler, the maximal size was calculated in cm^2, and a photograph was taken.

Biochemical parameters included complete blood cell count, erythrocyte sedimentation rate, C-reactive protein, glucose, creatinine, uric acid, total cholesterol, high density lipoprotein- (HDL-) cholesterol, low density lipoprotein- (LDL-) cholesterol, triglycerides, glycosylated hemoglobin, aspartate aminotransferase, alanine aminotransferase, and 24 h albuminuria.

Evaluation of metabolic control and adjustment of treatment were done as needed. Patients were reinforced about offloading their affected extremities, and adherence to treatment was assessed by requesting the empty tubes. Finally, possible harm including burning, redness, itching, and hypergranulation was monitored by physical examination and questioning. Unexpected adverse events were

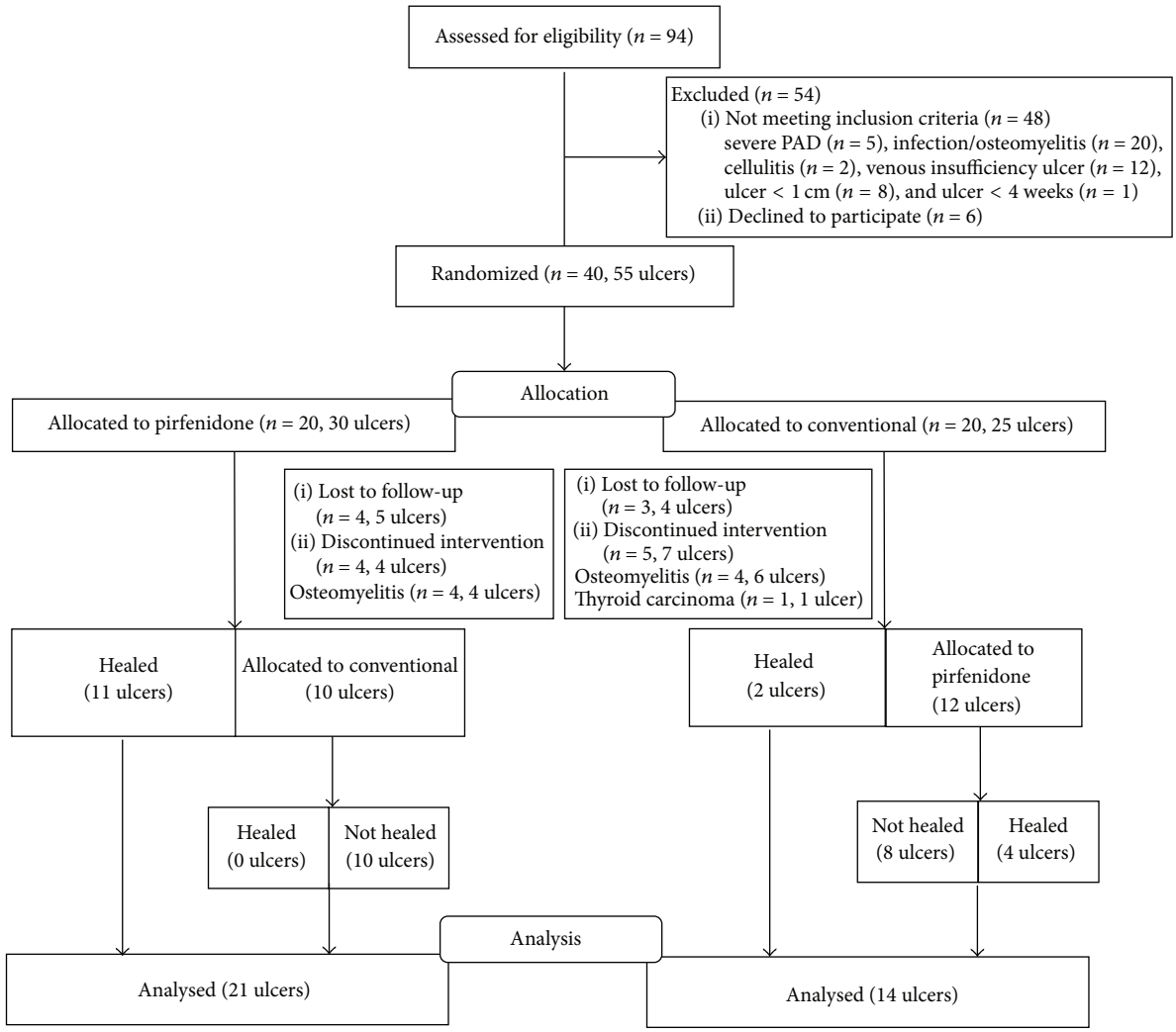

Figure 1: Flow diagram of patients during the study.

also recorded. Attribution to treatment was decided by an unblinded investigator.

2.4. Outcomes. Complete wound closure was defined as full epithelialization of the ulcer with absence of drainage. Percentage of closure was estimated by calculating the ulcer size in cm^2 at visit 1 and at visit 8. The size at the end of each period was subtracted to the initial ulcer size (week 1 and week 8) and the change was estimated and expressed as a percentage.

2.5. Sample Size. A sample size of 50 ulcers was calculated estimating a 25% change in ulcer size with pirfenidone treatment, with an alpha error of 5% ($Z_\alpha = 1.96$) and a beta error of 10% (power of 90%).

2.6. Randomization. Participants were randomly assigned in blocks to one of the two groups with the use of sealed envelopes using a random sequence numbers generator.

2.7. Statistical Analysis. The distribution of quantitative variables was analyzed with the Shapiro-Wilk test. Normal distributed variables were described as means and standard deviation. In the case of nonnormal distributed variables, median and interquartile ranges are used. Categorical variables were described as percentages and proportions. Differences between groups were evaluated with independent Student's t-tests or U Mann-Whitney tests, as appropriate. For categorical variables, chi-squared test was used. Statistical significance was considered with a two-sided P value < 0.05. The statistical program SPSS version 20 was used to perform the analysis.

3. Results

3.1. Participant Flow. Forty patients with 55 ulcers were randomized to pirfenidone ($n = 20$) or conventional ($n = 20$) treatment. The flow diagram of randomized patients is shown in Figure 1.

Table 1: Baseline characteristics of the studied population.

Variable	Group 1 ($n = 20$)	Group 2 ($n = 20$)	P
Male, number (%)	13 (65)	15 (75)	0.366
Age (years)	55.9 ± 14.2	54.7 ± 11.2	0.769
BMI, kg/m^2	25.3 [23.3–30.7]	29.0 [25.8–33.4]	0.048
Systolic blood pressure, mmHg	130 [115–140]	130 [120–140]	0.859
Diastolic blood pressure, mmHg	70 [70–80]	70 [70–85]	0.318
Time from DM diagnosis, years	18.3 [14.3–28.0]	15.3 [9.6–21.0]	0.107
Glucose, mg/dL	148 [110–196]	136 [108–191]	0.988
Creatinine, mg/dL	1.2 [.99–2]	0.95 [.86–1.3]	0.059
Albuminuria, mg/24 h	277.4 [27.4–758]	87.4 [11–739.6]	0.241
A1c, %	8.2 [7.2–8.4]	8.6 [7.1–9.5]	0.184
Triglycerides, mg/dL	135.5 [109–192]	133.5 [97.5–251]	0.930
Total cholesterol, mg/dL	172 [137–178]	159 [137–183]	0.988
HDL cholesterol, mg/dL	44.2 ± 13.5	41.8 ± 8.8	0.503
LDL cholesterol, mg/dL	93.9 ± 33.2	90 ± 34.1	0.733
ALT, U/L	17 [13–23]	18 [13–30]	0.837
AST, U/L	18 [17–24]	19.5 [14–22]	0.937
Uric acid, mg/dL	6.9 ± 1.5	6.5 ± 1.7	0.480
Hemoglobin, g/dL	12.7 [11.7–13.5]	13.5 [12.2–15.4]	0.349
Hematocrit, %	38 [36–41.5]	39.9 [36.7–45.9]	0.388
Platelets, K/μL	229.2 ± 54	239.2 ± 61.9	0.608
White blood cells, $\times 10^3$	7 ± 1.3	7.1 ± 1.2	0.868
ESR, mm/h	14 [5–34.5]	12 [6–32]	0.987
C-reactive protein, mg/dL	.28 [.18–.94]	.35 [.18–.93]	0.690

Data is expressed as mean ± SD or median [interquartile range]. BMI: body mass index calculated as weight in kilograms divided by the square of height in meters; DM: diabetes mellitus; A1c: glycated hemoglobin; HDL: high density lipoprotein; LDL: low density lipoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ESR: erythrocyte sedimentation rate.

3.2. Baseline Data. Participants' characteristics were not different between the groups except for the body mass index, which was slightly higher in the group assigned to receive conventional treatment first (29 vs 25.3 kg/m^2, $P = 0.048$). We do not consider that this slight difference in the body mass index may have affected the results. These data are presented in Table 1.

Baseline ulcer size and depth were not different between groups. However, according to the ABI classification, in group 1 there were more patients with PAD and in group 2 there were more patients with noncompressible arteries. We excluded patients with severe PAD and do not consider that these differences affected the results. Table 2 shows the baseline ulcer characteristics.

3.3. Outcomes. In the first eight weeks, 52.4% of the ulcers assigned to group 1 healed compared with 14.3% of group 2 ($P = 0.025$). After the crossover, the remaining 22 ulcers switched treatments and 30.8% of ulcers that received pirfenidone (during 8 to 16 weeks) healed compared with none in the conventional only group ($P = 0.081$). These figures are presented in Table 3.

At 8 weeks, the median percentage reduction in ulcer size was 100% [73–100] in group 1 compared with a 57.5% [28.9–74] reduction in group 2, $P = 0.011$ (Figure 2(a)). At 16 weeks, the median percentage reduction was 93% [42.7–100] in the pirfenidone group compared with a 21.8% [16–77.5] size reduction in the conventional group, $P = 0.050$ (Figure 2(b)).

Figure 3(a) shows an ulcer assigned to group 1 and Figure 3(b) an ulcer assigned to group 2.

3.4. Safety and Tolerability. No serious adverse events were observed during the course of the study in any treatment group. One patient in group 2 developed hypergranulation during pirfenidone treatment which resolved spontaneously. In group 2, a patient was diagnosed with thyroid carcinoma during conventional treatment and was eliminated from the study (Figure 1 and Table 4).

During the first 8 weeks, 10 ulcers (8 patients) developed osteomyelitis. Four ulcers (4 patients) were in group 1 and 6 ulcers (4 patients) in group 2; one of them had a supracondylar amputation. These patients were eliminated from the study (Table 4).

4. Discussion

This study demonstrates that pirfenidone added to conventional treatment is superior to conventional treatment alone.

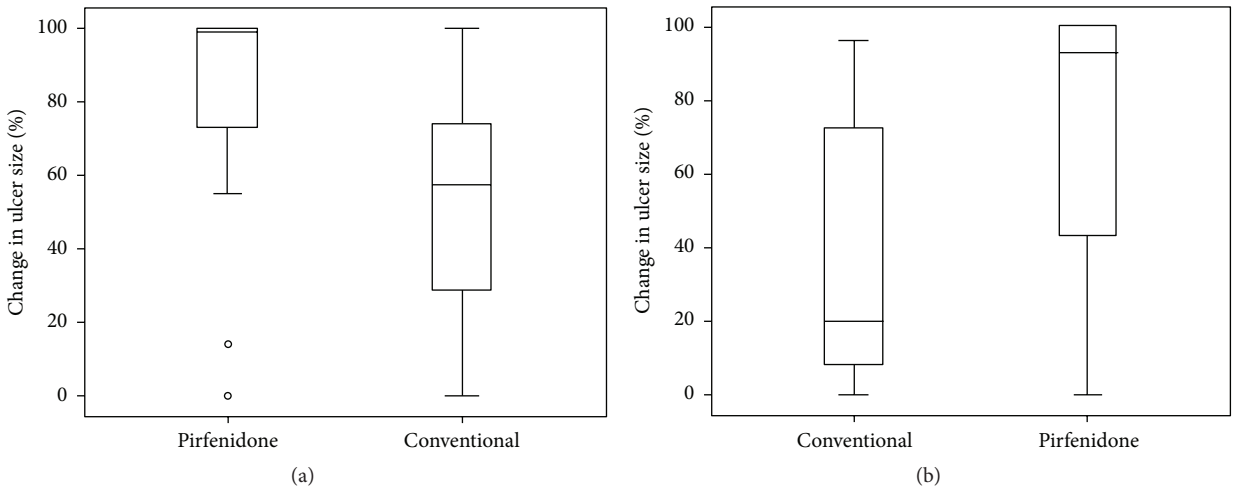

Figure 2: (a) Change in ulcer size expressed as percentage from baseline to 8 weeks. (b) Change in ulcer size expressed as percentage from 8 weeks to 16 weeks.

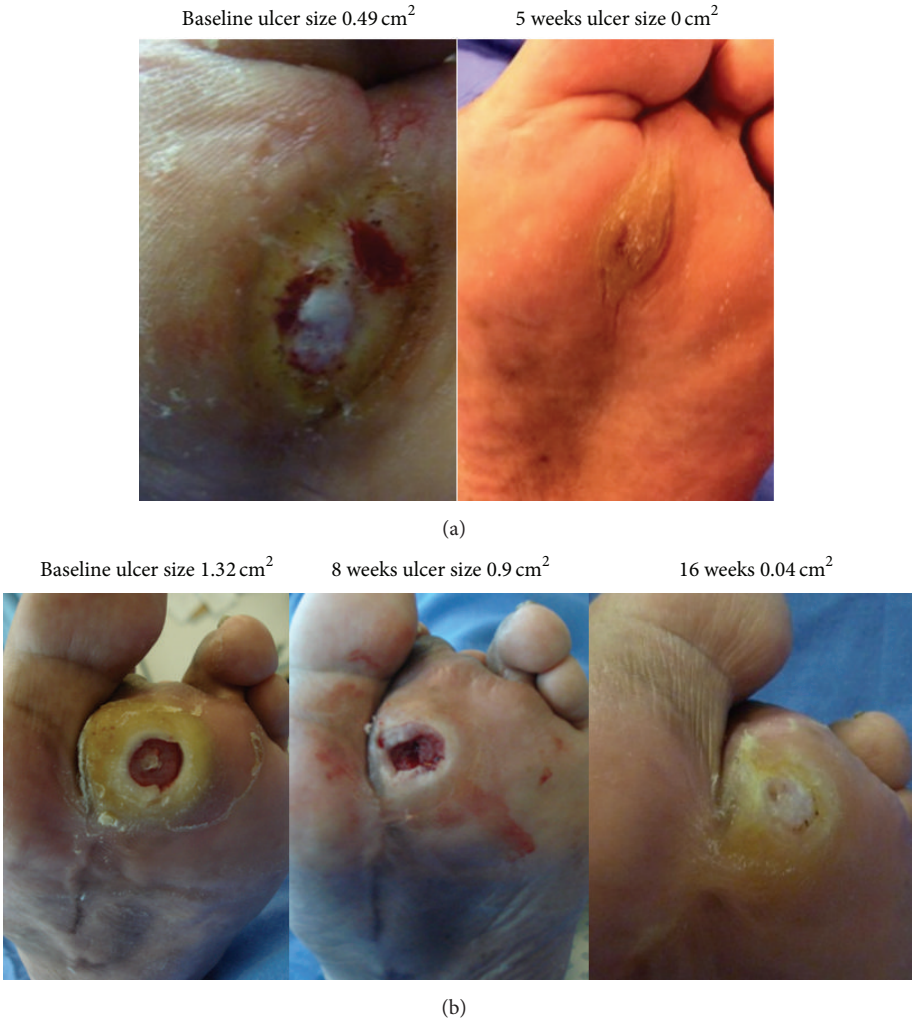

Figure 3: (a) Ulcer assigned to pirfenidone plus conventional treatment group during the first 8 weeks, that healed before crossover. (b) Ulcer assigned to conventional treatment only during the first 8 weeks, and crossover to pirfenidone plus conventional treatment.

Table 2: Baseline ulcer characteristics.

Characteristics	Total ($n = 35$)	Group 1 ($n = 21$)	Group 2 ($n = 14$)	P
Size, cm^2	1.32 [0.49–6.55]	0.75 [0.40–7.56]	1.40 [1.08–3.41]	0.630
Depth				0.955
Superficial	15 (42.9)	9 (42.9)	6 (42.9)	
Dermis, muscle, tendon	18 (51.4)	11 (52.4)	7 (50.0)	
All layers	2 (5.7)	1 (4.8)	1 (7.1)	
ABI right				0.406
Normal	7 (20)	5 (23.8)	2 (14.3)	
Noncompressible	28 (80)	16 (76.2)	12 (85.7)	
ABI left*				0.003
Normal	11 (32.4)	10 (50)	1 (7.1)	
PAD	3 (8.8)	3 (15)	0	
Noncompressible	20 (58.8)	8 (35)	13 (92.9)	
Depth				0.970
Superficial	15 (41.6)	9 (40.9)	6 (42.9)	
Dermis, muscle, tendon	18 (50)	11 (50)	7 (50)	
All layers	3 (8.3)	2 (9.1)	1 (7.1)	

Data expressed in median [interquartile range] or number (percentage).
ABI: ankle brachial index; PAD: peripheral arterial disease.
ABI was classified as follows: normal from >0.9–1.3, ≤0.9 PAD, <0.4 severe PAD, and >1.3 noncompressible vessel [11].
*In one patient left ABI could not be estimated due to history of amputation.

Table 3: Complete healing in the treatment groups.

	All ulcers N (%)	Pirfenidone N (%)	Conventional N (%)	P
Ulcer healing < 8 weeks (35 ulcers at the beginning)	13 (37.1)	11 (52.4)	2 (14.3)	0.025
Ulcer healing 8–16 weeks (22 ulcers at the beginning)	4 (17.4)	4 (30.8)	0	0.081

Treatment with pirfenidone decreased the size and increased the rate of complete healing of chronic noninfected diabetic foot ulcers. A greater number of ulcers achieved complete wound closure when receiving pirfenidone treatment.

Pirfenidone enhanced successful healing in addition to the standard ulcer care. A key component of the intervention was the weekly ulcer debridement and offloading the affected foot. Debridement enables removal of devitalized and necrotic tissue and promotes the beginning of the healing process [14]. The addition of pirfenidone accelerated the wound healing process.

Chronic diabetic foot ulcers represent a therapeutic challenge and their treatment involves debridement, frequent assessment, identification and treatment of infection, revascularization if indicated, and satisfactory offloading the foot [15].

Analysis of the evidence regarding the effectiveness of interventions to enhance healing of chronic diabetic foot ulcers is difficult. There are few controlled studies and the majority have methodological problems. There is not strong evidence to choose a specific dressing or topical medication in preference to another. Products designed to improve wound biochemistry and cell biology to promote wound healing demonstrate an ulcer healing rate between 40% and 80%. However, the evidence to support their use is not robust and further rigorously designed blinded trials are needed [8].

The results of this study are not generalizable due to the strict selection criteria. We excluded and eliminated patients with critical arterial insufficiency and infection to avoid confounders. Also, these conditions require specific and individualized treatment [16, 17]. A crossover design was chosen because otherwise it would not have been possible to control variables that could influence significantly the healing process such as ulcer size, ulcer depth, glycemic control, physical activity, and weight.

5. Conclusion

In summary, this study demonstrates that the addition of topical pirfenidone to conventional treatment significantly improves the healing of chronic diabetic noninfected foot ulcers.

Table 4: Adverse events.

	Group 1		Group 2	
	Weeks 0 to 7	Weeks 8 to 16	Weeks 0 to 7	Weeks 8 to 16
	Pirfenidone	Conventional treatment	Conventional treatment	Pirfenidone
Osteomyelitis	4 (eliminated)	0	6 (eliminated)	0
Hypergranulation	0	0	0	1
Thyroid carcinoma	0	0	1 (eliminated)	0
Total AE	4	0	7	1

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Prevalence and Risk Factors for Diabetic Lower Limb Amputation: A Clinic-Based Case Control Study

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Objective. The aim of the study was to evaluate the prevalence of and risk factors for lower limb amputation in a specialist foot clinic-based setting. *Methods.* A retrospective quantitative study was conducted, using clinical and biochemical profiles of diabetic foot patients attending the High Risk Foot Clinic at The Townsville Hospital, Australia, between January 1, 2011, and December 31, 2013. *Results.* The total study sample included 129 subjects, comprising 81 males and 48 females with M : F ratio of 1.7 : 1. Twenty-three subjects were Indigenous Australians, representing 17.8% of the study population. The average age of the cohort was 63.4 years ± 14.1 years [CI 90.98–65.89]. Lower limb amputation was identified as a common and significant outcome ($n = 44$), occurring in 34.1%, more commonly amongst the Indigenous Australians (56.5% versus 29.2%; $p = 0.94$, OR 0.94). Risk factors most closely associated with amputation included diabetic retinopathy ($p = 0.00$, OR 4.4), coronary artery bypass graft (CABG) surgery ($p = 0.01$, OR 4.1), Charcot's arthropathy ($p = 0.01$, OR 2.9), and Indigenous ethnicity ($p = 0.01$, OR 3.4). Although average serum creatinine, corrected calcium, and glycosylated haemoglobin A1c (Hba1c) levels were higher amongst amputees they were statistically insignificant. *Conclusions.* Lower limb amputation is a common outcome and linked to ethnicity and neurovascular diabetic complications amongst subjects with diabetic foot ulcer. Further research is needed to identify why risk of lower limb amputation seems to differ according to ethnicity.

1. Introduction

Diabetes and the diabetic foot ulcer (DFU) have made their mark in society, with the prevalence of diabetes being four times higher than all cancers combined [1]. Increased life expectancies have contributed significantly to this exponential rise, with diabetes now contributing to 9% of global mortality, equating to 4 million deaths per year [2, 3]. DFU occurs as a diabetic complication and involves a multifactorial pathogenesis including peripheral neuropathy as the primary causal factor, together with variable contribution from peripheral vascular disease (PVD), repetitive trauma, and superimposing foot infection [4, 5]. Infected DFU is a major cause of prolonged hospital admission and contributes over 90% of nontraumatic lower limb amputations (LLAs) [6, 7], which is more than a million amputations/year [8–10]. Whilst the 1990 St. Vincent Declaration to half LLAs in 5 years has failed [11], we have instead seen a 50% 5-year mortality rate amongst diabetic amputees [12–14].

The progressive rise of diabetes is likely to pose a significant burden on future society leading to an associated increase in diabetic amputations [15, 16]. Despite previous alert to the importance of early detection and management, prevention practices remain poor, with inconsistent patient follow-up and management compliance [17, 18]. As a result, subjects with DFU maintain poorer quality of life, with higher baseline depression rate, and 5-year mortality rates of up to 74% [19]. Existing studies have identified Indigenous ethnicity and presence of microvascular complications as contributing factors to poor DFU outcomes; however there is currently limited Australian evidence supporting this [20]. Furthermore there is no current data on the burden of either amputation or macrovascular outcomes amongst subjects with DFU in clinic setting in Australia which is home to

Table 1: Basic study characteristics and prevalence of adverse outcomes amongst the diabetic foot ulcer cohort.

Characteristic	Results			
Age	63.43 years ± 14.07 years [CI 60.98–65.89]			
Sex	Males (n = 81; 62.8%); females (n = 48; 37.2%)			
Ethnicity	Indigenous (n = 23; 17.8%); non-Indigenous (n = 106; 82.2%)			
Type of diabetes	Type 1 (n = 22; 17.1%); type 2 (n = 107; 82.9%)			
Type of ulcer	Ischaemic (n = 57; 44.2%); nonischaemic (n = 72; 55.8%)			
Clinical outcome	Total study sample	Ischaemic ulcer cohort	Nonischaemic ulcer cohort	p value, OR [CI]
Amputation	(n = 44)—34.1%	(n = 20)—35.1%	(n = 24)—33.3%	p = 0.84, 1.1 [0.52–2.25]
Minor amputation	(n = 35)—27.13%	(n = 15)—42.9%	(n = 20)—57.1%	p = 0.50, 0.9 [0.14–2.62]
Major amputation	(n = 9)—6.98%	(n = 5)—55.5%	(n = 4)—44.4%	p = 0.50, 1.5 [0.46–4.84]
Indigenous amputations	(n = 13)—56.5%	(n = 9)—69.2%	(n = 4)—30.8%	p = 0.94, 0.9 [0.17–5.07]
Ischaemic heart disease	(n = 61)—47.3%	(n = 31)—54.4%	(n = 30)—41.7%	p = 0.15, 1.7 [0.83–3.36]
Acute myocardial infarction	(n = 27)—20.9%	(n = 15)—26.3%	(n = 12)—16.7%	p = 0.18, 1.8 [0.76–4.20]
Cerebrovascular accidents	(n = 19)—14.7%	(n = 11)—19.3%	(n = 8)—11.1%	p = 0.19, 1.9 [0.71–5.13]
Chronic kidney disease	(n = 52)—40.3%	(n = 27)—47.4%	(n = 25)—34.7%	p = 0.20, 1.6 [0.78–3.23]
Dialysis	(n = 7)—5.4%	(n = 4)—7.0%	(n = 3)—4.2%	p = 0.48, 1.7 [0.37–8.09]
Peripheral vascular disease	(n = 94)—72.9%	(n = 44)—77.2%	(n = 50)—69.4%	p = 0.33, 1.5 [0.67–3.30]

amongst others Indigenous population at risk of diabetes. This study endeavoured to bridge these current knowledge gaps, with particular focus on determining prevalence of and identifying risk factors of limb amputations amongst patients with DFU attending a typical Australian regional diabetes foot clinic.

2. Methods

2.1. Eligibility Criteria. All patients attending the High Risk Foot Clinic (HRFC) at Northeastern Australia's Townsville Hospital between January 1, 2011, and December 31, 2013, were included in the study. Patients 18 years of age and over with a confirmed diagnosis of either type 1 or type 2 diabetes and coexisting DFU were included. Subjects under the age of 18 and those with nondiabetic foot ulcers (e.g., trauma-related, vasculitic, and neoplastic ulcers) were excluded from the study. Subjects who attended the clinic for other management purposes (e.g., podiatry reviews, nail pathology, and education about prevention) were also excluded.

2.2. Data Extraction. There were two main processes involved in data extraction, including gathering of clinical and biochemical data from patient profile. A retrospective chart audit was initially performed, focusing on the correspondence and outpatient attendance sections. The HRFC pro forma sheet was then used to collect information regarding the onset, duration, outcome, and type of ulcer defined as ischaemic or nonischaemic based on University of Texas Wound Classification System [21]. Furthermore, clinical information on the diagnosis and management of diabetes, coexisting macrovascular and microvascular complications, and medication lists of the subjects was recorded. We also obtained biochemical data using the hospital's pathology system, AUSLAB. Main parameters gathered included results of full blood count, urea and electrolyte studies, lipid profiles, Hba1c levels, and serum calcium, phosphate, and C-reactive protein levels. Average figures over the three-year period were calculated and included.

2.3. Data Analysis. SPSS software was utilised to perform data analysis. Basic descriptive and frequency analyses of the study sample were implemented to obtain demographic characteristics, period prevalence of clinical outcomes and diabetic complications, and mean age of the study population. In addition, a combination of nonparametric and chi-squared analyses was performed to identify differences in scaled data and rank risk factors associated with amputation, respectively. p value of less than 0.05 was considered statistically significant and together with odds ratios and confidence intervals was included in our results. All significant values were entered into binary logistic regression analysis and correction for multiple regression logistic testing was then conducted and factored in as part of our final results.

3. Results

3.1. Study Characteristics. A total of 129 subjects were included in the analysis of this study, with 62.8% being male (n = 81) and 37.2% female (n = 48) (refer to Table 1). The mean age of the study cohort was 63.4 ± 14.1 years [CI 60.98–65.89]. The Indigenous cohort comprised 17.8% (n = 23). Patients were categorised according to comparison groups of ischaemic (n = 57) and nonischaemic (n = 72) ulcers. The period prevalence of amputation within the study sample was 34.1% (n = 44), with 35.1% belonging to ischaemic (n = 20) and 33.3% nonischaemic (n = 24) cohorts. Amputation occurred more commonly at a rate of 56.5% amongst the Indigenous subjects, in comparison with 29.2% in the non-Indigenous group, with a significant difference amongst the ischaemic and nonischaemic ulcer cohorts (69.2% versus 30.8%). The mean age of amputation of 62.6 ± 12.5 years

Table 2: Summary of biochemical parameters amongst the study cohort.

Biochemical variable	Cohort		p value
	Ischaemic ulcers	*Nonischaemic ulcers*	
Average Hba1c level	8.5%	9.0%	$p = 0.06$
Average CRP level	38.6	72.7	$p = 0.14$
	Amputees	*Nonamputees*	
Average Hba1c level	9.0%	8.5%	$p = 0.44$
Average correct Ca^{2+} level	2.4 mmol/L	2.3 mmol/L	$^{*}p = 0.00$
Average serum creatinine level	156.2 μmol/L	121 μmol/L	$p = 0.19$
	Males	*Females*	
Average serum creatinine level	155 μmol/L	99.3 μmol/L	$^{*}p = 0.00$
Average eGFR level	57.4 mL/min	63.9 mL/min	$p = 0.22$
	Indigenous	*Non-Indigenous*	
Average white cell count	7.4	8.9	$^{*}p = 0.01$
Average albumin level	37	34.2	$p = 0.06$

*Statistically significant.

[CI 55.09–70.14] amongst the Indigenous cohort was similar to their non-Indigenous counterparts, mean age of 62.0 ± 11.5 years [CI 57.81–66.25]. Amputation rates were much higher amongst patients with PVD (39.4% versus 20%). Males got amputated more frequently (59.1%) compared to females (40.9%), although the mean age of male and female amputees differed minimally at 61.0 years and 63.9 years, respectively. Prevalence of ischaemic heart disease (IHD), acute myocardial infarcts (AMI), CABG surgery, strokes, and dialysis was significantly higher amongst ischaemic ulcer patients, occurring at overall rates of 54.4%, 26.3%, 15.8%, 19.3%, and 7.0%, respectively.

3.2. Biochemical Parameters amongst the Study Cohort. Using nonparametric analysis, corrected calcium levels were calculated to be higher amongst the amputee cohort (2.37 mmol/L versus 2.26 mmol/L ($p = 0.003$)) (refer to Table 2) though clinically nonsignificant with normal range 2.15–2.60 mmol/L. Similarly, although amputees were found to have higher serum creatinine (156.21 μmol/L versus 120.91 μmol/L) and Hba1c (9.0% versus 8.5%) levels, these results were statistically insignificant ($p = 0.19$ and $p = 0.44$). Indigenous subjects had lower average white cell counts ($p = 0.005$) and higher albumin levels ($p = 0.058$). Males tended to have poorer kidney function than females, with average creatinine levels of 155.03 μmol/L versus 99.25 μmol/L ($p = 0.003$) and eGFR levels of 57.94 mL/min versus 63.90 mL/min ($p = 0.22$).

3.3. Clinical Risk Factors Associated with Amputation. Chi-squared analysis identified diabetic retinopathy ($p = 0.00$, OR 4.4 [2.15–12.75]), Indigenous background ($p = 0.01$, OR 3.1 [1.17–9.16]), Charcot's arthropathy ($p = 0.01$, OR 2.9 [1.38–9.29]), longer diabetes duration, defined as 15 years or longer ($p = 0.03$, 2.2 [1.00–4.86]), dyslipidaemia ($p = 0.04$, 3.4 [0.94–12.38]), neuropathy ($p = 0.03$, 3.3 [1.05–10.26]), PVD ($p = 0.04$, 2.6 [1.03–6.55]), and CABG surgery ($p = 0.01$, OR 4.1 [1.81–30.76]) to be significantly associated with increased risk of amputation, amongst others (refer to Table 3). Binary logistic regression analyses identified retinopathy, CABG surgery, Charcot's arthropathy, and Indigenous background as the most significant risk factors for amputation (refer to Table 4). Clinical parameters that fell short of statistical significance include smoking history ($p = 0.49$), dialysis ($p = 0.62$), diabetes type ($p = 0.46$), male sex ($p = 0.53$), multiple-ulcer history ($p = 0.15$), previous history of DFUs ($p = 0.06$), and ulcer type ($p = 0.84$) and grade ($p = 0.93$).

4. Discussion

4.1. Prevalence of Lower Limb Amputations. In this clinic-based case control study we have demonstrated prevalence of amputation in our study population to be 34.1%, a figure that is considerably higher in comparison with others' findings of 15.4% to 21.4% [22, 23]. On the other hand our report conforms to Miner and Kirsner and Amogne et al. report of higher rate of LLA in their respective diabetic foot clinic populations [13, 24]. The high prevalence might be related to inclusion of the population at the highest risk of the disease and its complications [25]. In this study, Indigenous Australians were found to be at greater risk of diabetic LLA, which is in keeping with others' observation [20, 26]. Furthermore, whilst there was a marginal difference in amputation between ischaemic and nonischaemic cohorts in the overall group, amputations related to ischaemic ulcers were more than double amongst the Indigenous subgroup. Essentially, the prevalence of amputation amongst our subjects stood at comparatively higher numbers and occurred predominantly amongst Indigenous subjects with ischaemic ulcers.

4.2. Risk Factors for Lower Limb Amputations. We identified numerous clinical factors that correlate with higher amputation risk, most in keeping with previous literature, in addition to new, undocumented parameters. The most significant contributing factors were diabetic retinopathy, CABG surgery, Charcot's foot, and Indigenous ethnicity. Whilst Ndip et al.

Table 3: Summary of risk factors for lower limb amputation amongst the cohort using chi-squared analysis.

Risk factor	p value, OR [CI]
Acute myocardial infarction	$p = 0.20$, 1.8 [0.74–4.16]
Antihypertensive medications	$p = 0.28$, 1.8 [0.62–5.32]
Cellulitis	* $p = 0.00$, 3.3 [1.54–7.21]
Cerebrovascular accidents	$p = 0.07$, 2.5 [0.93–6.67]
Charcot's arthropathy	* $p = 0.01$, 2.9 [1.29–6.70]
Chronic kidney disease	$p = 0.27$, 1.5 [0.72–3.16]
Coronary artery bypass graft surgery	* $p = 0.01$, 4.1 [1.29–13.17]
Depression	$p = 0.05$, 2.2 [0.98–5.10]
Dialysis	$p = 0.62$, 1.5 [0.32–6.94]
Dyslipidaemia	* $p = 0.04$, 3.4 [0.94–12.38]
eGFR < 45 mL/min	$p = 0.08$, 2.1 [0.91–4.73]
Foot antibiotics	* $p = 0.04$, 2.3 [1.03–4.98]
Gastrooesophageal reflux disease (GORD)	$p = 0.34$, 1.5 [0.67–3.15]
Haemoglobin < 8 g/dL	$p = 0.46$, 1.7 [0.42–6.64]
Hba1c > 7.5%	$p = 0.92$, 1.1 [0.40–2.73]
Hypertension	$p = 0.41$, 1.6 [0.50–5.43]
Hypoalbuminaemia	$p = 0.73$, 0.9 [0.37–1.99]
Indigenous ethnicity	* $p = 0.01$, 3.1 [1.25–7.92]
Infection severity (mod-severe)	* $p = 0.02$, 0.4 [0.18–0.89]
Insulin treatment	$p = 0.12$, 1.9 [0.85–4.28]
Ischaemic heart disease	$p = 0.24$, 1.6 [0.75–3.24]
Ischaemic ulcer type	$p = 0.84$, 1.1 [0.52–2.25]
Longer duration of diabetes	* $p = 0.03$, 2.2 [1.00–4.86]
Male sex	$p = 0.53$, 0.8 [0.37–1.66]
Multiple ulcers	$p = 0.15$, 0.6 [0.25–1.25]
Nephropathy	$p = 0.15$, 1.7 [0.82–3.56]
Neuropathy	* $p = 0.03$, 3.3 [1.05–10.26]
Obesity	$p = 0.09$, 0.5 [0.23–1.13]
Osteomyelitis	* $p = 0.00$, 3.9 [1.54–10.07]
Peripheral vascular disease	* $p = 0.04$, 2.6 [1.03–6.55]
Previous history of ulcers	$p = 0.06$, 2.1 [0.98–4.41]
Retinopathy	* $p = 0.00$, 4.4 [1.99–9.59]
Smoking history	$p = 0.49$, 0.8 [0.37–1.60]
Statin therapy	$p = 0.06$, 2.7 [0.95–7.78]
Type of diabetes	$p = 0.46$, 1.4 [0.56–3.65]
Wound classification	$p = 0.93$, 1.1 [0.30–3.78]

∗ denotes being statistically significant.

Table 4: Risk factors associated with lower limb amputation in the study population [logistic regression analysis].

Risk factor	p value, OR [CI]
Coronary artery bypass graft surgery	$p = 0.01$, 7.5 [1.81–30.76]
Indigenous ethnicity	$p = 0.02$, 3.3 [1.17–9.16]
Charcot's arthropathy	$p = 0.01$, 3.6 [1.38–9.29]
Retinopathy	$p = 0.00$, 5.2 [2.15–12.75]

provide data linking diabetic retinopathy to increased risk of DFU development [27], ours is the first study to identify retinopathy not only as a contributing factor, but also as the most significant factor leading to amputation amongst DF patients, accentuating the importance of early detection and management of diabetic complications. McEwen et al. found no association with retinopathy but reported a 2-3-fold amputation risk amongst patients with diabetic neuropathy [28], results that are consistent with ours, which isolate neuropathy and dyslipidaemia as significant risk factors for amputation. We identified PVD with increased risk of adverse outcome, also supported by current literature, which labels it as a causal factor for amputation and mortality amongst diabetics [29–32]. We have additionally extended existing knowledge by highlighting CABG surgery as a novel risk factor for amputation, which supports the role of both microvascular and macrovascular disease as significant risk factors for LLA in subjects with diabetic foot ulcer.

Interestingly, there has been conflicting data on the role of ethnicity in the outcome of DFUs. Whilst multiple studies found unremarkable figures in amputation rates amongst a multiracial cohort [27, 28], Lavery et al. found a significant difference in both prevalence and ulcer severity contributing to amputation amongst African American cohorts in other parts of the world [33]. Correspondingly, we have found Indigenous ethnicity to be amongst the strongest contributing factors in our cohort, who were almost twice as likely to undergo an amputation. The higher prevalence of amputations in the group of Indigenous Australians could be attributed to a genetic predisposition or to a socioeconomic status that drives the patients to present late for clinical care. This result is supported by previous Australian data stating that Indigenous Australians are known to develop diabetes and its associated metabolic complications at a younger age [24, 34]. As the first study to be conducted in Northeastern Australia which hosts some of the largest Indigenous peoples nationwide, our results hold considerable significance for the local population, given that longer diabetes duration was flagged as a contributor for adverse outcome and highlights the need for earlier detection and management amongst Indigenous Australians [32, 35, 36].

Our results identified other risk factors of LLA in subjects with DFU including Charcot's arthropathy, a history of osteomyelitis, and severity of foot infection or cellulitis requiring antibiotic treatment. Our result was in keeping with the previously reported 30% rate of amputation in subjects with Charcot's arthropathy, placing DFU patients at a 12 times higher lifetime risk of amputation [37, 38]. Similarly, in keeping with our findings, Wukich et al. have linked history of cellulitis and moderate-to-severe foot infection to amputation [39]. There is notably no current evidence suggesting use of antibiotics to prevent infections in subjects with DFU at risk of LLA in spite of our findings of antibiotics use preceding amputation. The tropical climate of Northeastern Australia is a likely contributor to this

association, resulting in increased rates of bacterial skin and soft tissue infections requiring ongoing antimicrobial treatment. Moreover, a new association was also found with depression, suggesting that psychological health can be an indicator of healing and recovery in physical illness. Given that the DFU is known to have a biopsychosocial impact on its patients, this information accents the importance of a multidisciplinary team approach in treating the individual as a whole and calls for additional research in the area.

In contrast to others' observation, we found no association between renal disease and amputation risk, specifically with CKD and dialysis, all of which have previously been linked with amputation [35, 40]. Amongst our dialysis cohort, all seven subjects were male, with one having Indigenous background. These characteristics were similar to Lavery et al.'s study, whose dialysis cohort mainly focused on a non-Hispanic Caucasian population, yet whilst they identified more limb amputations amongst their renal disease subgroup, we found no significant association between the two [41]. This could be explained by the nature of our study cohort, which focused on DFU patients with concurrent renal disease rather than a specific CKD or dialysis population, thereby missing a number of diabetic LLA patients that either did not meet the criteria or failed to attend the HRFC. Intensive glycaemic control has been established as an important prognostic healing factor and found to reduce diabetes-related mortality [42, 43]. However when discussed in linkage with lower extremity complications, some studies report a 20% higher risk of amputation amongst subjects with Hba1c levels above 7.5% similar to our findings but in contrast to Winkley et al. who reported Hba1c levels below 7.5% as having higher mortality and increased risk of amputation [31, 32, 44]. In light of this conflicting report further research is required to characterise the findings.

The period prevalence of IHD and AMI amongst the study cohort was similar to others' report indicating high prevalence of cardiovascular disease and its complications amongst the DF cohort [45]. CKD and dialysis were present in 40.3% and 5.4%, which was once again lower compared to international data and again likely due to the fact that ours was not a renal-specific cohort. We did, however, establish higher rate of PVD (72.9%) compared to Setacci et al., who reported a period prevalence of up to 30% amongst the diabetic cohort [46], which reinforces the role of PVD in contributing to amputation. Furthermore, 14.7% of the population had a background history of strokes, with no previous studies available with which to make comparisons. Our results have appropriately highlighted that the high rates of adverse multisystemic, vascular outcomes amongst patients with the DF necessitate a multidisciplinary approach in treatment delivery.

4.3. Strengths and Limitations. Major study strength included the data extraction process, which utilised a wide range of demographic, clinical, and biochemical data to formulate an extensive analysis to support the study aims. This study implemented a highly focused study cohort with a good representation of Indigenous subjects to evaluate and compare our results with international data. It is important to note that there is a limitation to retrospective studies in general. Observations derived from such studies may contain some missing information and thus may serve as a stimulus to further prospective work to clarify findings. The present work must be interpreted in the knowledge of the defects inherent in such studies. Nevertheless, our result is in agreement with other reports [20, 22, 47].

5. Conclusions

We have documented high prevalence of lower limb amputation in our study population. In keeping with our hypothesis, the ischaemic ulcer cohort demonstrated higher rates of adverse clinical outcomes, including IHD, strokes, and renal disease. Numerous known and novel demographic and clinical risk factors were coupled with amputation, the most significant of them being Indigenous ethnicity, diabetic retinopathy, Charcot's arthropathy, and CABG surgery. Whilst there were no significant differences in amputation prevalence between the ischaemic and nonischaemic groups in the overall cohort, Indigenous subjects with ischaemic ulcers were amputated much more frequently. Extended research in the local area is encouraged to study factors leading to selectively higher amputation rate in the Indigenous population. We have made a huge development in identifying predisposing characteristics amongst DF patients, but our knowledge is not yet comprehensive to enable us to prevent limb amputation in the high risk diabetic population.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Assessment of Left Ventricular Structural Remodelling in Patients with Diabetic Cardiomyopathy by Cardiovascular Magnetic Resonance

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Background. Diabetic cardiomyopathy (DCM) is always accompanied with alteration of left ventricular structure and function. The aims of this study were to assess the structural remodelling in patients with DCM by cardiovascular magnetic resonance (CMR) and correlation of structural remodelling with severity of DCM. *Methods*. Twenty-five patients (53.8±8.8 years, 52.0% males) with DCM and thirty-one normal healthy controls (51.9 ± 13.6 years, 45.2% males) were scanned by CMR cine to assess function and structure of left ventricular. Length of diabetic history and results of cardiac echocardiography (E', A', and E'/A') were also measured. *Results*. Compared with normal controls group, DCM group was associated with significantly increased ratio of left ventricular mass at end diastole to end-diastolic volume (MVR) ($P < 0.05$) and no significant difference was in mass at end diastole ($P > 0.05$). The ratio correlated with both length of diabetic history and echocardiographic Doppler tissue imaging E' (all $P < 0.05$). *Conclusions*. CMR can be a powerful technique to assess LV remodelling, and MVR may be considered as an imaging marker to evaluate the severity of LV remodelling in patients with DCM.

1. Introduction

Type 2 diabetes mellitus (T2DM), one of the most common chronic diseases, affects nearly four hundred million people in all countries [1]. As estimated [2], there will be a seventy-percent increase in adults with T2DM in developing countries and a nearly 20% increase in developed countries between 2010 and 2030. Diabetic cardiomyopathy is considered as myocardial dysfunction in almost two-thirds of patients with T2DM, which occurred independently of coronary heart disease (CHD), valvular dysfunction, and hypertension [3, 4]. The reasons for diabetes-related adverse myocardial alteration are not clear. But LV remodelling, especially concentric remodelling, is an emerging candidate mechanism. LV concentric remodelling, a kind of patterns of LV remodelling, is characterized by an increased ratio of LV mass to volume (MVR) and normal LV myocardial mass index at ED [5].

For a long time, LV remodelling has been assessed by echocardiography [6–8]. But there are several limitations for echocardiography, such as limited view and susceptible reproducibility, when compared with cardiovascular magnetic resonance (CMR). With high resolution and signal to noise ratio, CMR is being regarded as the golden standard for the assessment of cardiac function and structure [9, 10]. Importantly, the accuration and reproducibility of volume and mass measurement were well tested in inter- and intra-center [11].

There has not been widely accepted in normal range of MVR measured by CMR, let alone in patients with DCM. The special aims of this study were to investigate the

LV remodelling in DCM patients by CMR and correlating between LV remodelling and diabetic history.

2. Patients and Methods

2.1. Patients. Twenty-five patients with diabetic cardiomyopathy were recruited from June 2015 to March 2016 at Southwest Hospital of Third Military Medical University. The inclusion criteria included (a) first diagnosis of type 2 diabetes mellitus with the criteria from World Health Organization [12], (b) decreased left ventricular diastolic function, the ratio of early diastolic mitral annular velocity E' to late velocity A' ($E'/A' < 1$) by echocardiographic Doppler tissue imaging [13], and (c) no history of hypertension and suspicious coronary heart disease. The following were exclusion criteria: (a) estimated glomerular filtration rate (eGFR) $\leq$ 30 mL/min/1.7 m^2, (b) age < 30 years, or >70 years, and (c) other standard CMR contraindications, such as claustrophobia or any history of body metal implant. Two candidates quitted because of high heart rate during being scanned. Therefore, a total of 25 patients were enrolled. At the same time, thirty-one age- and gender-matched normal healthy controls from community were recruited by their own volition to this study. All patients and normal controls were gave the informed consent, which were conducted in accordance with the Declaration of Helsinki (1964). This study was approved by the Institutional Review Board of Southwest Hospital (the first affiliated hospital of Third Military Medical University).

2.2. Demography Exam. All DCMs and normal controls were measured height, weight to yield body surface area (BSA) and body mass index (BMI), and systolic and diastolic blood pressure. Serum glucose, HbA1c, pancreas function (insulin, c peptide), renal function (blood urea nitrogen (BUN), creatinine, and cystatin C), lipids profile (triglycerides, total cholesterol, high-density and low-density lipoprotein cholesterol (HDL, LDL)) were analyzed. Myocardium zymogram examination (aspartate aminotransferase (AST), lactate dehydrogenase (LDH), α-hydroxybutyrate dehydrogenase (α-HBDH), creatine kinase (CK), ischemia modified albumin (IMA)), and markers of myocardial damage (troponin and myoglobin) were assessed in 86% patients and controls. Urine microalbumin (U-MTB) was also measured in the majority of participants (79%).

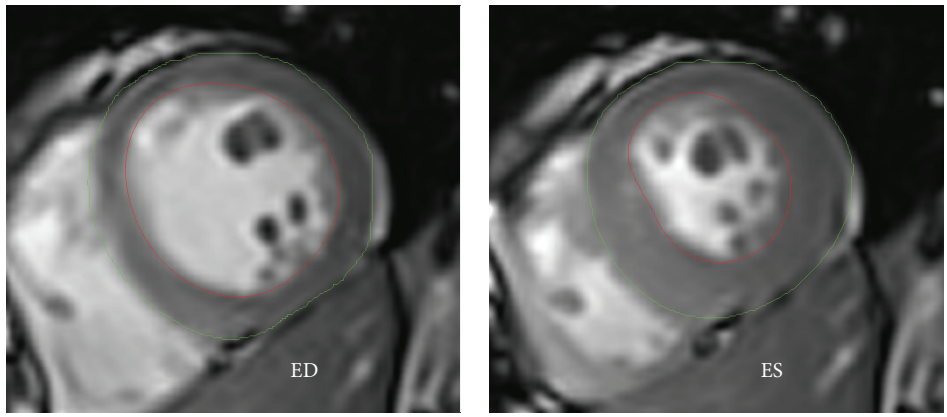
2.3. Echocardiography. All DCMs were performed transthoracic echocardiography at rest by a commercial ultrasound machine (Philips IE33 color Doppler scanner, the Netherlands) and a matching transducer (S5-1 heart probe). According to the American Society of Echocardiography's recommended guidelines [13], assessment of LV diastolic function was carried out by the cardiac echocardiographic Doppler tissue imaging (DTI) at an apical four-chamber view with the sample volume placed at mitral valve annulus. Early diastolic septal mitral annular velocity E' (passive left ventricular filling), late velocity A' (atrial contraction), and their ratio (E'/A') were measured to yield the LV diastolic function. All images were stored in Digital Imaging and Communications in Medicine (DICOM) format for online analysis.

2.4. Cardiovascular Magnetic Resonance Protocol. All DCMs and healthy controls were scanned by CMR on a 3.0 T whole-body system (Trio Tim, Siemens Healthcare, Erlangen, Germany) in the supine position and head first with a matching 12-channel matrix body coil and electrocardiographic gating instrument. Breath held in expiration when necessary. The localizing images, including standard four-chamber (left ventricular, left atrium, right ventricular and right atrium), two-chamber (left ventricular and left atrium), and short-axis images, were obtained.

Segmented short-axis cine images were acquired with two-dimensional (2D) steady-state free precession (SSFP) pulse sequence (TR/TE 59.22/1.45 ms, FOV 400 × 325 mm^2, flip angle 50°, matrix size 256 × 179, voxel size 2.2 × 1.6 × 6.0 mm^3, slice thickness 6 mm, spacing between slices 1.5 mm, and 25 phases per cardiac cycle) [14]. To cover the whole LV, the position line of the first basal cine images was localized through both the insertion point of mitral valve into septum and free wall in both standard four-chamber and two-chamber slice. The last short-axis cine was localized at apex. The position lines of all other slices were put to parallel to the first line. Because of difference in length of left ventricular among all participants, the number of their own slices ranged from 8 to 14. Similarly, due to difference in heart rate among them, time of holding breath ranged from 6 to 10 seconds.

2.5. Cardiovascular Magnetic Resonance Analysis. Cine images were analyzed offline using the dedicated commercial available software (Argus, Siemens Healthcare, Erlangen, Germany) by two experienced observers (one with 10 years of experience in CMR and the other 6 years of experience) without knowing any information of the participants. After all short-axis cine images of LV were loaded, the end-systolic (ES) phase and end-diastolic (ED) phase were visually identified as the smallest and largest chamber area separately on the middle slice. The myocardial endocardial and epicardial contours of LV were carefully manually delineated on all images (Figure 1) to acquire the absolute function parameters including end-systolic volume (ESV), end-diastolic volume (EDV), stroke volume (SV), ejection fraction (EF), cardiac output (CO), myocardial mass at ED, and normalized function parameters including ESV/BSA (ESVi), EDV/BSA (EDVi), SV/BSA (SVi), cardiac index (CI), myocardial mass/BSA (massi), and ratio of myocardial mass to volume of LV [15]. Moderator bands and papillary muscles were carefully assigned in the lumen of LV.

2.6. Statistical Analysis. Categorical variables were showed as percentage. Kolmogorov-Smirnov test was used to test normality of variables, and variables that did not fit normal distribution were summarized as median and quartile. Variables, which were continuously and normal distribution, were presented as mean and standard deviation (SD). Independent sample *t*-test was used to evaluate the differences in function parameters and demographic variables between the DCMs



(a) (b)

Figure 1: Epi- and endocardial contours on end-diastolic phase (a) and end-systolic phase (b).

and normal. Mann-Whitney U test was used to evaluate the differences in biochemical variables if the variables did not fit normal distribution or standard variance was heterogeneity. The relationship between MVR and diabetic history was analyzed by Pearson's or Spearman's method. Statistical tests were two-tailed, and $P < 0.05$ was considered to reach statistical significance. Statistical analysis was performed on the commercial available software (SPSS for Windows 21.0, Inc, Chicago, IL, USA).

3. Results

3.1. Results of Demographic and Biochemical Characteristics. Results of demographic and biochemical characteristics were showed in Table 1.

Twenty-five patients (13 male, age 53.8±8.8 years, diabetic history 5.9 ± 3.9 years) with DCM, and thirty-one normal controls (14 male, age 51.9 ± 13.6 years) were studied. Fasting blood glucose and glycated hemoglobin significantly increased in DCMs. Insulin and C peptide were also elevated in DCMs. DCMs were also accompanied by higher triglycerides, HDL, and aspartate aminotransferase (AST). IMA and troponin were higher in DCMs, which showed possible impairment of myocardium related to T2DM. Age, gender, weight, height, BSA, BMI, blood pressure, and renal function (BUN, creatinine, cystatin C) were similar in the two groups (all $P > 0.05$).

3.2. Alteration of Left Ventricular Geometry and Function. Parameters of LV function and structure were summarized in Table 2.

The LV EDVi approximately decreased by 10% in DCMs (60.1 ± 7.7 versus 66.6 ± 10.6 mL/m^2, $P = 0.032$). There was no significant difference in LV massi at ED between the two groups (53.7 ± 9.8 versus 51.5 ± 8.9 g/m^2, $P = 0.378$). MVR increased by 15% in DCMs (0.90 ± 0.20 versus 0.79 ± 0.15 g/mL, $P = 0.025$; Table 2, Figure 2), suggesting that there was significant LV concentric remodelling. MVR did not show correlation with systolic blood pressure, diastolic blood pressure, age, height, weight, BMI, or BSA by separately bivariate Pearson's correlation test (All $P > 0.05$).

The LV ESVi was also smaller in DCMs (24.6 ± 5.9 versus 28.6 ± 6.9 mL/m^2, $P = 0.023$). DCM patients were similar in SVi, CI, and EF to normal controls (All $P > 0.05$), which revealed that systolic function of LV did not impair in DCMs. Echocardiography DTI showed that DCMs were associated with decreased E′/A′ (mean 0.80 ± 0.09).

3.3. The Relationship between Concentric Remodelling and Diabetic History. Pearson correlation analysis showed that MVR was positively correlated with the length of diabetic history ($r = 0.472$, $P = 0.017$, Figures 3 and 4). Stepwise multivariable linear regression showed that the length of diabetic history was the only independent predictor of LV MVR (normalized $\beta = 0.472$, $P = 0.017$; demographic variable, age, height, weight, BMI, BSA, and systolic and diastolic blood pressure). Spearman correlation showed that MVR was negatively correlated with echocardiography DTI E′ ($\rho = -0.435$, $P = 0.030$), although the correlation between MVR and E′/A′ did not reach statistical significance.

4. Discussion

Diabetic cardiomyopathy is regarded as T2DM-related myocardial dysfunction, which is independently of CHD, valvular dysfunction, and hypertension [3, 4]. In agreement with previous reports [16, 17], DCM was accompanied by changed LV geometry and function. Using CMR cine we show here that (1) DCM is accompanied by LV concentric remodelling, (2) MVR is associated with length of diabetic history, and (3) MVR is correlated with cardiac echocardiography DTI E′, indicating impaired LV diastolic function.

As reported in previous study [18–20], there is insulin resistance in patients with T2DM, in other words, pancreas islet secretes more insulin and C peptide, which causes hyperinsulinemia. In our study, DCMs were associated with hyperinsulinemia, higher C peptide, and metabolic syndrome, manifesting increased triglycerides and decreased

Table 1: Demographic characteristics.

	DCM ($N = 25$)	Control ($N = 31$)	P value
Anthropometry			
Age, y	53.8 ± 8.8	51.9 ± 13.6	0.533
Diabetic history, y	5.9 ± 3.9		
Male, %	52.0	45.2	0.611
Height, m	1.62 ± 0.07	1.61 ± 0.07	0.634
Weight, kg	63.8 ± 11.6	61.0 ± 10.5	0.336
BMI, kg/m^2	24.1 ± 2.9	23.4 ± 3.1	0.362
BSA, m^2	1.68 ± 0.18	1.65 ± 0.17	0.457
Systolic blood pressure, mmHg	122.2 ± 10.0	119.3 ± 11.5	0.324
Diastolic blood pressure, mmHg	81.6 ± 6.4	83.5 ± 7.7	0.333
Biochemical exam			
Urine microalbumin, mg/dL	0.3 [0.1–5.0]	1.2 [0.6–1.8]	0.177
Blood urea nitrogen, mmol/L	5.8 [4.8–7.0]	6.0 [5.1–6.9]	0.716
Creatinine, umol/L	68.7 ± 21.9	64.1 ± 16.9	0.381
Cystatin C, mg/L	0.77 [0.66–0.88]	0.75 [0.64–0.86]	0.594
AST,	22.0 [17.8–29.5]	29.5 [23.2–35.4]	**0.008**
LDH, IU/L	201.0 ± 45.3	197.7 ± 46.5	0.809
α-HBDH, IU/L	128.4 ± 31.6	131.2 ± 38.7	0.799
CK, IU/L	97.4 [69.9–123.5]	86.0 [65.4–108.4]	0.421
Ischemia modified albumin, U/mL	81.9 ± 7.5	73.6 ± 5.9	**0.001**
Total cholesterol, mmol/L	5.7 [4.8–6.9]	5.3 [4.9–6.0]	0.205
Triglycerides, mmol/L	2.4 [1.5–4.3]	1.3 [0.9–2.0]	**0.004**
HDL, mmol/L	1.2 [1.0–1.4]	1.4 [1.1–1.7]	**0.026**
LDL, mmol/L	3.5 [2.8–4.3]	3.3 [3.1–3.7]	0.703
Glucose, mmol/L	8.6 [7.2–11.1]	5.3 [4.9–5.8]	**0.000**
Glycated hemoglobin, %	6.9 [6.3–9.1]	5.6 [5.4–5.8]	**0.000**
Troponin, 10^{-3} ug/L	8 [5–12]	5 [3–6]	**0.003**
Myoglobin, ng/mL	27.6 [22.3–49.4]	30.5 [22.4–38.5]	0.739
Insulin, uIU/mL	16.4 ± 7.0	12.1 ± 4.3	**0.022**
C peptide, ng/mL	1.7 [1.2–2.3]	1.1 [0.8–1.4]	**0.007**
Echocardiography			
Doppler mitral annular velocity E'/A'	0.80 ± 0.09		

Table 2: Left ventricular geometry and function.

	DCM ($N = 25$)	Controls ($N = 31$)	P value
LV end-diastolic volume index (EDV), mL/m^2	60.1 ± 7.7	66.6 ± 10.6	**0.032**
LV end-systolic volume index (ESV), mL/m^2	24.6 ± 5.9	28.6 ± 6.9	**0.023**
LV stroke volume index (SV), mL/m^2	36.5 ± 4.7	38.0 ± 5.4	0.279
LV ejection fraction, %	60.1 ± 6.7	57.5 ± 5.3	0.102
Cardiac index (CI), $L/(min*m^2)$	2.8 ± 0.4	2.7 ± 0.5	0.749
LV mass index, g/m^2	53.7 ± 9.8	51.5 ± 8.9	0.378
LV mass to LV end-diastolic volume, g/mL	0.90 ± 0.20	0.79 ± 0.15	**0.025**

HDL. IMA, as a novel biomarker of tissue ischemia, was higher in diabetic patients than normal controls [21, 22], and elevated IMA levels also predict a subclinical cardiovascular disease in T2DM [23–25]. Our study showed that patients with DCM were associated with increased IMA, which indicated that myocardial tissue might suffer T2TM-related tissue ischemia. Our research was consistent with previous studies. Troponin, a biomarker of myocardial damage, are widely used to evaluate situation of patients with acute coronary syndromes. Nowadays, large cohort study [26] and case-control study [27] considered the cardiac troponin as independent predictor of major adverse cardiovascular events in T2DM patients. In this study, increased troponin parameters were found in patients with DCM.

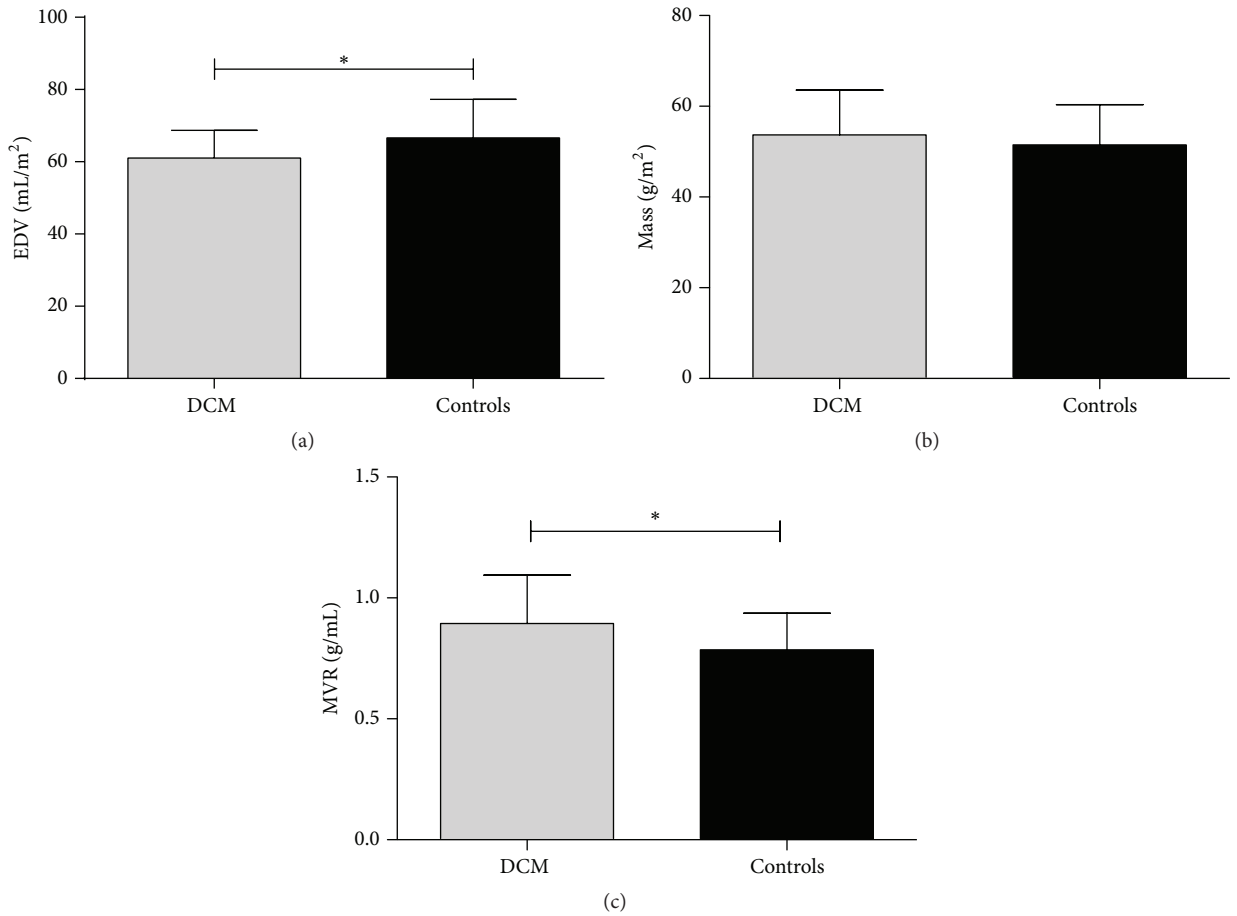


Figure 2: Differences in left ventricular geometry between DCMs and controls: (a) EDV, (b) myocardial mass at ED, and (c) MVR. $*$ means that $P < 0.05$.

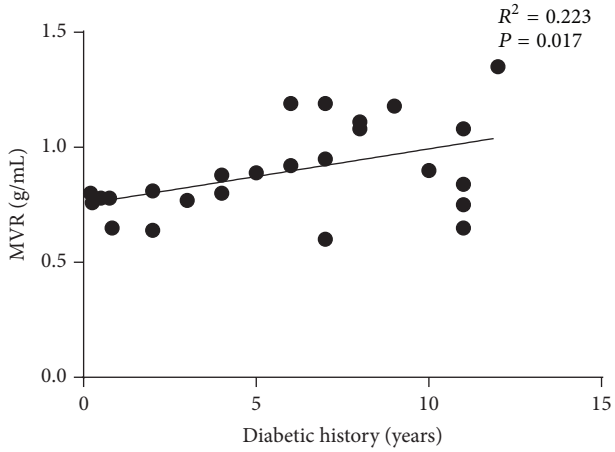


Figure 3: Relationship between length of diabetic history and MVR.

LV concentric remodelling, a kind of patterns of LV remodelling, was characterized by an increased ratio of LV mass to volume and normal LV myocardial mass index at ED [5]. For a long time, LV remodelling has been assessed by echocardiography. Due to limited view and susceptible reproducibility, echocardiography might not be a powerful technique when compared with CMR. CMR, with high spatial resolution and signal to noise ratio, is widely used to assess cardiac function and structure and being regarded as the golden standard. Therefore, CMR may be the better technique to assess MVR than echocardiography. Many kinds of disease may cause LV concentric remodelling. Previous studies have reported diabetes-related LV concentric remodelling [16, 28]. In our study age- and gender-matched normal healthy controls, in the absence of CHD, hypertension, and other cardiovascular disease, were enrolled in the same time. In this study, MVR of normal group was 0.79 ± 0.15 g/mL, which was consistent with previous studies with large population (totally 741 subjects) [29] and small population [16]. However, it is smaller than the value of MVR derived from another small study [28] and multiethnic study of atherosclerosis [30]. This is like the fact that the population, enrolled in the latter large study, was not highly selected. Participants with hypertension and even diabetes were included; therefore the results should not be considered as real range of healthy subjects. So large study with highly selected normal healthy population should be conducted to confirm the values of MVR in healthy subjects. The values of MVR in patients with DCM were not well-defined. In this study, MVR of DCM

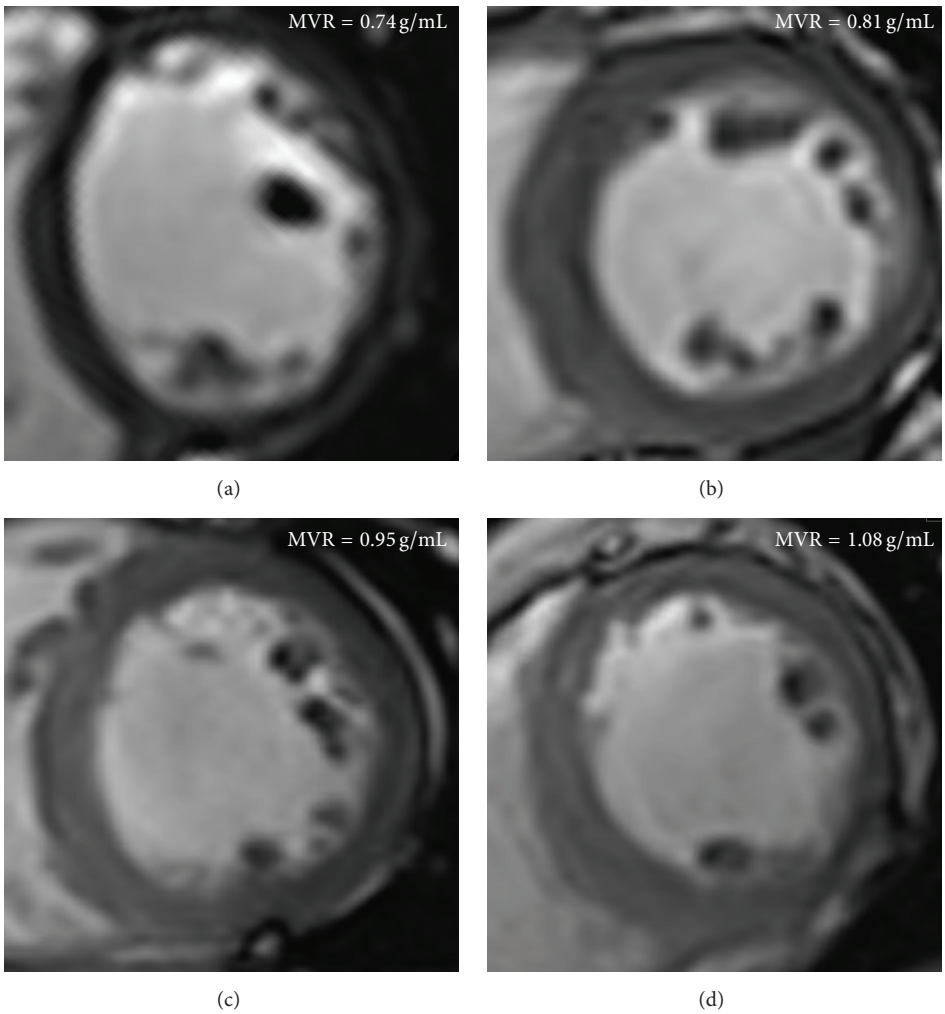


Figure 4: Representative examples of cardiac cine images in control and DCMs. (a) Control, 63 years old, female, EDVi = 69.9 mL/m^2, massi = 51.4 g/m^2, MVR = 0.74 g/mL. (b) DCM (2 years), 60 years old, male, EDVi = 63.1 mL/m^2, massi = 51.0 g/m^2, MVR = 0.81 g/mL. (c) DCM (7 years), 40 years old, male, EDVi = 66.3 mL/m^2, massi = 63.0 g/m^2, and MVR = 0.95 g/mL. (d) DCM (11 years), 57 years old, EDVi = 53.7 mL/m^2, massi = 58.0 g/m^2, and MVR = 1.08 g/mL.

group was 0.90 ± 0.20 g/mL, which is consistent with the value of the study [16], but a little smaller than the value of the study [28]. Both the two studies enrolled relatively small objects. Considering the heterogeneity of T2DM, large and highly selected patients should be recruited to discover the mystery of MVR in DCM.

It is intriguing that MVR was correlated with length of diabetic history, not age, blood pressure, changed AST, IMA, triglycerides, HDL, insulin, C peptide, glucose, and even HbA1c. Transient hyperglycemia can make persistent impairment to cell by disrupting signal feedback loop [31]. HBA1c, biomarker of time-averaged glucose level, and hyperglycemia cannot manifest the severity of long-term accumulated damage to myocardium. Neither the elevated troponin nor IMA can manifest the severity of long-term accumulated damage to myocardium. The relationship between length of diabetic history and MVR may prompt us that MVR may be considered as a biomarker of long-term effect of T2DM to myocardium, which should be confirmed by large cohort or cross section studies. What is more, decreased cardiac echocardiography DTI E′ and E′/A′ have been widely accepted as the marker of impaired LV early diastolic function [13]. In this study, we also found that E′ was correlated with MVR. Both of them indicated us that MVR of LV increased with diabetic progression, shown as length of diabetic history and impaired LV diastolic function. Taking together, MVR may be considered as an imaging marker to assess the severity of DCM.

There were some limitations in our study. First, the number of recruited patients and normal controls was small, and this study should be considered as preliminary. Large cohort or cross-section studies should be conducted to confirm the influences of diabetic history on LV remodelling. Second, patients only with GFR ≥ 30 mL/min/1.7 m^2 were recruited, and this inhomogeneity of patients may introduce a bias.

5. Conclusion

CMR can be a powerful technique to assess LV remodelling, and MVR may be considered as an imaging marker to evaluate the severity of LV remodelling in patients with DCM.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Yongning Shang and Xiaochun Zhang contributed equally to this paper.

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Review Article

Urinary Biomarkers in the Assessment of Early Diabetic Nephropathy

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Diabetic nephropathy (DN) is a frequent and severe complication of diabetes mellitus (DM). Its diagnosis in incipient stages may allow prompt interventions and an improved prognosis. Towards this aim, biomarkers for detecting early DN can be used. Microalbuminuria has been proven a remarkably useful biomarker, being used for diagnosis of DN, for assessing its associated condition—mainly cardiovascular ones—and for monitoring its progression. New researches are pointing that some of these biomarkers (i.e., glomerular, tubular, inflammation markers, and biomarkers of oxidative stress) precede albuminuria in some patients. However, their usefulness is widely debated in the literature and has not yet led to the validation of a new "gold standard" biomarker for the early diagnosis of DN. Currently, microalbuminuria is an important biomarker for both glomerular and tubular injury. Other glomerular biomarkers (transferrin and ceruloplasmin) are under evaluation. Tubular biomarkers in DN seem to be of a paramount importance in the early diagnosis of DN since tubular lesions occur early. Additionally, biomarkers of inflammation, oxidative stress, podocyte biomarkers, and vascular biomarkers have been employed for assessing early DN. The purpose of this review is to provide an overview of the current biomarkers used for the diagnosis of early DN.

1. Introduction

Diabetic nephropathy (DN) represents an important cause of chronic kidney disease (CKD) that frequently leads to end stage renal disease (ESRD). Diabetes mellitus (DM) is a frequent disease and DN is one of its main complications. It is appreciated that up to 40% of the patients with type I and type II DM present DN [1]. In Western countries, diabetes is a leading cause of chronic kidney disease frequently leading to chronic renal replacement therapy (RRT) due to ESRD [2].

Taking into account the increased incidence of both DM and of DN, the detection of early DN is of paramount importance, in order to provide appropriate therapy that prevents or slows evolution towards ESRD.

Biomarkers play an important role in the early detection of DN. Among them, the best known is microalbuminuria. At the same time, microalbuminuria represents a marker of the generalized endothelial dysfunction present in DM, linking renal involvement with cardiovascular and cerebral impairment.

In time, it has been demonstrated that microalbuminuria reflects not only glomerular injury but also tubular lesions, filtered albumin being reabsorbed at tubular level. Additionally, new biomarkers have been studied in order to identify tubular lesions in DM.

The new tubular biomarkers have been detected in both type 1 and type 2 DM early renal dysfunction that precedes microalbuminuria. At present, the assessment of early DN

involves numerous biomarkers. They span the period of normoalbuminuria that precedes microalbuminuria but also the evolution of renal involvement during microalbuminuria and macroalbuminuria.

Until they are universally accepted they are analyzed in relationship with the levels of albuminuria, especially of microalbuminuria.

At present, markers of inflammatory and oxidative processes accompanying DM and DN are also being assessed.

Since literature abounds in studies on markers highlighting renal dysfunction in different stages of the evolution of DM, we decided to restrict our study to the early phase of DN.

An update of the urinary biomarkers used in early DN is useful for establishing their role in the early diagnosis of this disease, with subsequent prophylactic and therapeutic implications. We insist on urinary biomarkers because they are easily drawn, which allows population screening, and because they can detect tubular lesions, which occur very early in DM.

Proteomics is an additional tool offering great prospects in DN assessment.

The origin of the biomarkers employed for assessing renal involvement in DM is diverse. Some of the biomarkers are constitutive elements of the nephron, such as markers at

(i) epithelial cell (podocyte) level, for example, nephrine and podocalyxin [3];

(ii) glomerular basement membrane level: collagen and laminin [4];

(iii) endothelial (VEGF) [5];

(iv) tubular cell level, for example, NGAL, NAG, and KIM [6].

Some have mixed origin; they can originate both in tubular cells and in podocytes, for example, angiotensinogen [7, 8].

Some are derived from the circulation, for example, transferrin, ceruloplasmin, and immunoglobulins G and M. They pass into the urine because of glomerular lesions which result in increased permeability for plasma proteins.

There are several classifications addressing the diversity of urinary biomarkers in DM.

Matheson classifies the biomarkers according to both their origin and the pathologic processes impairing the nephron: kidney damage, oxidative stress, and inflammation:

(i) biomarkers of renal dysfunction,

(ii) inflammatory biomarkers (cytokines and chemokines),

(iii) oxidative stress biomarkers [9].

Another classification belongs to Hong and Chia who present 3 categories of biomarkers:

(i) glomerular,

(ii) tubular,

(iii) other proteins [10].

It should be noted that products of metabolism in DM are also eliminated in the urine, and they can trigger toxic effects, for example, advanced glycation end products (AGE).

Since we will frequently refer to microalbuminuria in presenting other biomarkers used in studying lesions of the nephron, namely, of the glomerulus and of the tubules, we will first present the main observations regarding microalbuminuria in diagnosing DN.

Recent literature uses new terms, like moderately increased albuminuria for microalbuminuria and severely increased albuminuria for macroalbuminuria. However, the classical terms of microalbuminuria and microalbuminuria continue to be in wide use, as they are more practical. This is why we will prefer them in the present paper.

Urinary biomarkers use in assessment of early diabetic nephropathy are presented in Table 1.

2. Microalbuminuria (Moderately Increased Albuminuria) in Type 1 DM

Microalbuminuria usually begins 5–10 years after the onset of type 1 DM [11].

Kidney biopsy examination in patients with type 1 DM and microalbuminuria most frequently finds normal histological aspects. However, DN lesions were found in a small number of patients [12].

According to McKenna and Thompson, microalbuminuria is predictive element of the future development of end stage renal disease [13].

Microalbuminuria can regress towards normoalbuminuria, it can persist as such, or it can progress towards albuminuria [14, 15].

The evolution of microalbuminuria towards macroalbuminuria is usually related to arterial hypertension and reduced GFR, an important part being generally played by risk factors [16].

Persistent microalbuminuria is related to future development of end stage renal disease and to cardiovascular risk [13].

It should be noted that diminution of GFR usually occurs after the development of microalbuminuria, but there are situations when even normoalbuminuria is accompanied by diminution of the GFR [17].

3. Microalbuminuria (Moderately Increased Albuminuria) in Type 2 DM

Microalbuminuria is an important biomarker in type 2 DM, being frequently used in population-based screenings.

Regarding the prevalence of microalbuminuria in type 2 DM patients we highlight a review of Newman et al. of 28 studies on 10,294 patients. They found microalbuminuria in 26% of the patients with ten-year duration of the disease [18].

A study on 24,000 patients found that Asian and Hispanic patients with type 2 DM present more often microalbuminuria (43%) than whites (33%) [19].

In China, Shanghai, microalbuminuria has a prevalence of 41% among patients with type 2 DM [20].

Hypertensive patients with type 2 DM present microalbuminuria more frequently [21].

Table 1: Urinary biomarkers in the assessment of early diabetic nephropathy.

Glomerular biomarkers		Tubular biomarkers
Transferrin	*Microalbuminuria* The main marker in current use	Neutrophil gelatinase-associated lipocalin (NGAL)
Immunoglobulin G		Alpha-1-microglobulin
Ceruloplasmin		Kidney injury molecule-1 (KIM-1)
Type IV collagen		N-acetyl-β-D glucosaminidase
Laminin		Angiotensinogen
Glycosaminoglycans		Cystatin C
Lipocalin-type prostaglandin D synthase		Liver-type fatty acid binding protein
Fibronectin		Nephrine
Podocytes-podocalyxin		Heart fatty binding protein
Vascular endothelial growth factor/VEGF		Advanced glycation end products
Inflammatory biomarkers	Other new markers under study	Oxidative stress biomarkers
Tumor necrosis factor alpha	Retinol binding protein-4	8-Oxo-7,8-dihydro-2′-deoxyguanosine
Orosomucoid	Vitamin D binding protein	
	Heme oxygenase-1	
	Periostin	
	Alpha klotho	
	Microvesicle-bound dipeptidyl peptidase IV	
	MicroRNA	
	Adipokinesine alpha-2 glycoprotein	

Microalbuminuria can have a variable evolution. It can regress towards normal values, it can progress towards macroalbuminuria, or it can remain unchanged. In a study on 216 patients, Araki et al. found, after a 6-year follow-up, regression of microalbuminuria in 51% cases and progression to severely increased albuminuria in 28% cases [22].

The risk of progression to severely increased albuminuria is higher in patients with microalbuminuria as compared to patients with normoalbuminuria [18].

The diminution of GFR is also higher in patients with severely increased albuminuria than in those with microalbuminuria [23].

Glycemic control, ACE inhibitors, and ARBs for blood pressure control play an important role in the evolution of microalbuminuria. It should be mentioned that microalbuminuria has been considered a glomerular biomarker. To date, emerging data point to the role of the tubules in producing microalbuminuria [24, 25]. As such we did not include this marker among glomerular biomarkers but approached it separately, according to its potential role as both a glomerular and tubular biomarker.

3.1. Glomerular Biomarkers

3.1.1. Urinary Transferrin. Transferrin is a protein with a molecular weight of 76.5 KDa. Because of its low molecular weight and its less ionic load it filters easily through the glomerular barrier [26].

As increased urinary transferrin was found in type 2 DM normoalbuminuric patients, concomitantly with urinary ceruloplasmin and immunoglobulin G, preceding microalbuminuria, it could be considered a biomarker of early DN [27].

In microalbuminuric patients the levels of urinary transferrin increase [28]. They also increase in patients with type 2 DM with vascular complications: coronary artery disease, diabetic retinopathy, and so forth [29].

Patients with initial high levels of urinary transferrin excretion will develop microalbuminuria more frequently than those with normal levels [30].

3.1.2. Urinary Immunoglobulin G. It is an anionic plasma protein with a molecular weight of 150 KDa that crosses the glomerular barrier with difficulty [31].

As presented above, urinary IgG can be secreted before the stage of microalbuminuria, concomitantly with increased values of urinary transferrin, urinary ceruloplasmin, and urinary orosomucoid [27].

Increased elimination of urinary IgG could thus predict microalbuminuria in DM patients [27].

3.1.3. Urinary Ceruloplasmin. Ceruloplasmin is a copper-transporting serum protein. It is filtered with difficulty at glomerular level because it is more negatively charged [32].

It was also found in some type 2 DM patients with normoalbuminuria, arguing in favour of its use for early detection of renal lesions, even prior to albuminuria: ceruloplasmin could have in type 2 DM patients a DN predictive effect, similar to urinary transferrin and urinary immunoglobulin G [27]. According to Yamazaki et al. the

urinary ceruloplasmin excretion rate (CER) and clearance of ceruloplasmin increase in parallel with the progression of albuminuria [33].

In fact, in type 2 DM patients there could exist a parallelism between increased values of urinary transferrin, urinary immunoglobulin G, and urinary ceruloplasmin [27].

We conclude that urinary ceruloplasmin could be used for the early diagnosis of DN [34].

3.1.4. Type IV Urinary Collagen. Type IV collagen is a component of the glomerular basement membrane and of the mesangial matrix [35].

In DN, lesions are produced both at glomerular capillary level and at mesangial level. Its excretion in urine might serve as early indicator of renal injury associated with DN [36].

Increased levels of type IV urinary collagen are reported for normoalbuminuric patients with type 1 DM. It could be a biomarker used for the early diagnosis of DN [37]. Other authors also report increased excretion of type IV collagen and of laminin in patients with type 1 DM [23].

High urinary type IV collagen excretion was also reported in normoalbuminuric patients with impaired glucose tolerance [38].

Urinary type IV collagen could reflect morphological renal alterations in patients with type 2 DM. A relationship between the severity of histological lesions and urinary type IV collagen was reported in patients with type 2 DM [39].

Type IV urinary collagen is considered to be a specific indicator of early DN [40].

It could also allow both detection of early DN in patients with type 2 DM and differential diagnosis with glomerulonephritis, where its levels are low [41].

3.1.5. Urinary Laminin. Laminin is a component of the glomerular basement membrane. Its urinary excretion is increased in normoalbuminuric type 2 DM patients, being correlated with NAG (N-acetyl-beta-D-glucosaminidase) and alpha-1-microglobulin excretion. Concomitantly increased excretion of type IV collagen is found [4].

3.1.6. Urinary Glycosaminoglycans. Glycosaminoglycans are components of the glomerular basement membrane as well as of the extracellular matrix. In DM alterations of these occur, the excretion of glycosaminoglycans being increased, even in normoalbuminuric patients [42].

Glycosaminoglycans are also present at the level of the tubular basement membrane. Urinary glycosaminoglycans are associated with other tubular biomarkers, for example, Tamm-Horsfall protein, which expresses a distal tubular dysfunction in diabetic patients [43].

3.1.7. Lipocalin-Type Prostaglandin-D Synthase (L-PGDS). It is a biomarker related to lesions of the glomerular capillary walls and reflects their increased permeability. It is mainly considered to predict renal lesions, being less relevant as an early marker of DN [44].

3.1.8. Urinary IgM and Urinary Fibronectin. These were studied only sporadically, without sufficient data to support their use as markers of early DN.

Urinary fibronectin excretion is significantly increased in DM patients only if they present microalbuminuria [45]. IgM is an indicator of impaired kidney function [46].

Although the use of urinary glomerular biomarkers has not become current practice yet, glomerular biomarkers have been reported in some normoalbuminuric patients, leading to the conclusion that albuminuria might not represent the most sensitive glomerular biomarker. However, their clinical applicability needs to be confirmed in high-quality validation studies [31].

3.2. Tubular Biomarkers. DN is manifested mainly by well-known glomerular lesions. The aforementioned biomarkers are identified already precociously early in early DN. Tubulointerstitial lesions are associated with glomerular injury during DN [47]. Tubular biomarkers have shown that tubular dysfunction can be present early in DN, sometimes preceding glomerular injury. These biomarkers have proven highly sensitive as compared to microalbuminuria, which is considered the gold standard biomarker of DN. In fact, presently, microalbuminuria is regarded not only as a glomerular biomarker, but also as a tubular one.

3.2.1. Neutrophil Gelatinase-Associated Lipocalin (NGAL). NGAL—neutrophil gelatinase-associated lipocalin—is a glycoprotein present in the kidneys at tubular cell level and is considered to be protective against renal damage [48].

Urinary NGAL is a biomarker used in assessing tubular lesions in DM, its increased values being present even in the initial phases of the disease, namely, in normoalbuminuric patients [49].

Thus, in type 1 DM high urinary NGAL can precede microalbuminuria [50, 51].

Urinary NGAL had high values in type 2 DM patients with normoalbuminuria, increasing progressively in patients with microalbuminuria and macroalbuminuria. The values of KIM-1 (kidney injury molecule-1) increased in parallel, indicating an early and progressive lesion [52].

However, Fu et al. reported in type 2 DM patients who present hyperfiltration and increased values of urinary NGAL, as well as of urinary KIM-1, as compared to the values of patients with normal GFR [53].

Urinary NGAL shows the precocity of tubular lesions in patients with prediabetes [54].

Urinary NGAL in type 2 DM patients could have a role in predicting the evolution of disease [55].

3.2.2. Urinary Alpha-1-Microglobulin. Urinary alpha-1-microglobulin is a serum protein with low molecular weight (27-kDa), which allows it to get easily filtered through the glomerular capillary wall. Once it arrives in the proximal tubule, alpha-1-microglobulin is reabsorbed and metabolized. Tubular dysfunction leads to alteration of reabsorption with increased excretion in the urine [56].

In a cross-sectional study, Hong et al. analyzed 590 type 2 DM patients and found that 33.6% patients with

normoalbuminuria presented increased values of urinary alpha-1-microglobulin, a fact that could be explained by tubular injury that precedes the occurrence of microalbuminuria, being a more sensitive and an earlier urinary biomarker [57]. However, alpha-1-microalbuminuria can be absent in some patients with albuminuria [57]. This is why assessments of alpha-1-microglobulin are associated with the assessment of other urinary biomarkers, urinary albumin included.

Petrica et al. reported high values of urinary alpha-1-microglobulin in normoalbuminuric patients, a fact pleading for an early tubular injury in type 2 DM in this stage. They did not find correlations between urinary alpha-1-microglobulin, beta-2 microglobulin, and the albumin/creatinine ratio with plasma asymmetric dimethyl-arginine. This could plead for dissociation between tubular and endothelial dysfunction [58].

Alpha-1-microglobulin in early stages of DM could also have a role in predicting DN [59]. It is in fact an inexpensive biomarker of early diagnosis of DN [60].

3.2.3. Urinary KIM-1 (Kidney Injury Molecule-1). KIM-1 is a transmembrane glycoprotein located at the level of the proximal tubular cells. It is eliminated in urine in case of injury at this level. It is a sensitive biomarker used with good results in acute kidney injury [61].

Petrica et al. reported in normoalbuminuric type 2 DM patients high values of urinary KIM-1, which indicates lesions of the proximal tubule in early stages of the disease. Patients with microalbuminuria have higher urinary KIM-1 values than those with normoalbuminuria [62].

de Carvalho et al. reported in type 2 DM normoalbuminuric patients high values of KIM-1, these values increasing progressively in patients with microalbuminuria and macroalbuminuria. NGAL values studied concomitantly presented similar evolutions [52].

Moreover, KIM-1 presents higher elimination in type 2 DM patients with hyperfiltration than in patients with normal glomerular filtration. NGAL has a similar evolution. These biomarkers—KIM-1 and NGAL—could plead for a deleterious lesional effect of hyperfiltration on the proximal tubule [53].

Nielsen et al. however could not demonstrate a value of urinary KIM-1 that could be predictive of the evolution of glomerular function (GFR) in patients with type 1 DM [63].

According to Nielsen et al. it has no prognostic utility in type 2 DM patients either [64].

3.2.4. Urinary N-Acetyl-β-D glucosaminidase (NAG). NAG is an enzyme located in the lysosomes of proximal tubular cells [65].

In case of dysfunction, namely, of injury of proximal tubular cells, NAG is eliminated into the urine in higher quantities, being a sensitive tubular biomarker. This can precede the appearance of microalbuminuria in type 1 DM [66].

Elevated serum Cys C levels and urinary NAG activities were found only in normoalbuminurics, not in controls. In addition, elevated urinary ALP and LDH activities were also found in microalbuminurics [67].

Other authors, like Ambade et al., did not find that urinary NAG has clinical significance as an early biomarker of DN [68].

In type 2 DM urinary NAG excretion increases proportionally to the duration of diabetes. It occurs much earlier than albuminuria. NAG can be considered an early tubular biomarker [69].

Assal et al. consider that urinary NAG is the most sensitive biomarker for detecting early damage in diabetic patients [70].

3.2.5. Urinary Angiotensinogen. The renin angiotensin aldosterone system (RAAS) is involved in the pathogenesis of DN. The constitutive elements of RAAS are present at kidney level, defining a local RAAS.

Urinary angiotensinogen can represent a biomarker for the activation of RAAS in DM [71].

High urinary angiotensinogen precedes in type 1 DM patients the occurrence of microalbuminuria [72]. This could have a predictive role in normotensive type 1 DM patients [73].

Urinary angiotensinogen in normoalbuminuric type 2 DM patients is higher than in controls and it increases progressively in microalbuminuric and especially in macroalbuminuric patients [73].

Urinary angiotensinogen can be considered an early biomarker of DN [72].

In type 2 DM patients, urinary angiotensinogen is correlated with alpha-1-microglobulin [8].

Kim et al. did not confirm these observations in a study on type 2 DM patients. They found that the values of urinary angiotensinogen are not different from those of the controls, in normoalbuminuric and microalbuminuric type 2 DM patients, but higher values were described in macroalbuminuric patients [74].

These observations point to the need of further studies necessary for the validation of this biomarker.

Increased urinary angiotensinogen could represent a risk factor in renal and cardiovascular complications [75].

Since activation of RAAS could intervene in the evolution of DN, administration of ACE-I is recommended.

At the same time, urinary angiotensinogen could be a marker for assessing the renoprotective effects of alogliptin to type 2 DM patients [76].

3.2.6. Cystatin C. It is a low molecular weight protein having the role of cysteine protease. Cystatin is produced by the nucleated cells in the body [77].

It is filtered at glomerular level and is reabsorbed in the tubules. Cystatin is used for evaluating renal function. Assessment of GFR by means of cystatin C is considered to be a method that is not influenced by body mass, being comparable and even better than methods using serum creatinine [78].

Serum cystatin is also considered a sensitive biomarker as it detects minor glomerular injury [79].

Urinary cystatin C indicates tubular injury. It increases early in diabetes and prediabetic nephropathy [80].

Patients with microalbuminuria present higher values of urinary cystatin C than those without microalbuminuria, urinary cystatin C having a predictive role for the progression of diabetic nephropathy (DN) [81].

Urinary cystatin C level could be an independent factor for identifying renal dysfunction in type 2 DM patients with normoalbuminuria, including patients with GFR <60/mL/min/1.73 m^2 [77]. Uslu et al. find a significant positive correlation between serum cystatin C, urinary NAG, lacticodehydrogenase, alkaline phosphatase activities, and serum creatinine levels [67].

Serum and urinary cystatin C are useful biomarkers for assessing early nephropathy in type 2 DM [77].

3.2.7. L-FABP (Liver-Type Fatty Acid Binding Protein). Urinary L-FABP is a protein with low molecular weight expressed in the cytoplasm of human proximal tubular cells [82]. It is also expressed at liver level.

Increased urinary L-FABP was found in type 1 DM patients who presented normoalbuminuria, having a predictive role regarding the progression towards microalbuminuria and of microalbuminuria towards macroalbuminuria [83].

Patients with type 2 DM with normoalbuminuria also presented high levels of urinary L-FABP, this protein being considered as a useful biomarker for diagnosing early diabetic nephropathy. In fact, urinary L-FABP has been confirmed as a tubular biomarker by the Ministry of Health and Welfare in Japan [82].

The L-FABP factor is also related to the severity of DN. The values of urinary L-FABP increase with the decline of renal function [84].

Although some authors, like Chou et al., do not ascribe a predictive role to urinary L-FABP in type 2 DM patients [85], others, like Panduru et al., consider that urinary L-FABP is an independent predictor of the progression of DN [86].

3.2.8. Nephrinuria. Nephrine is a transmembrane protein in the structure of the slit diaphragm [87].

In DM podocyte dysfunction is present. DN is considered a podocytopathy [88]. Injury of the slit diaphragm leads to nephrinuria.

Nephrinuria can occur in some type 1 DM patients prior to microalbuminuria [89]. Nephrinuria was also reported in some normoalbuminuric type 2 DM patients [62, 90].

Nephrinuria is related to podocyte injury representing a biomarker of early glomerular injury [91].

Dysregulation of nephrine in podocytes in DN could lead to nephrinuria in normoalbuminuric patients, preceding microalbuminuria [92].

In albuminuric patients, nephrinuria is positively correlated with albuminuria and negatively correlated with GFR, being a biomarker of DN in other phases of DM as well.

Podocyte impairment in DM involves not only nephrine but also other podocyte elements, for example, VEGF. Thus, in normoalbuminuric DM patients nephrinuria is correlated with urinary elimination of VEGF [62].

Tubular biomarkers seem to play an important role in the early diagnosis of DN. They manage to show, in most cases, that microalbuminuria does not represent a reliable biomarker for diagnosing incipient lesions of DN. However, up to now, none of these biomarkers has been established as gold standard for the identification of early DN.

3.3. Markers of Inflammation. DM is accompanied by chronic inflammatory processes affecting the whole body, the kidneys included. Mediators of inflammation, like cytokines and chemokines, are present in these processes. Some of them are useful as markers of inflammation.

3.3.1. Tumour Necrosis Factor Alpha (TNF Alpha). Urinary TNF alpha presents in type 2 DM patients with microalbuminuria and macroalbuminuria higher values than in patients with normoalbuminuria. Urinary TNF alpha is correlated with urinary NAG, a marker of tubular lesions [93].

Cherney et al. analyzed in a complex study on normoalbuminuric type 1 DM patients forty-two urinary cytokines/chemokines. They found that the urinary level of IL6 and IL8, the platelet-derived growth factor, and RANTES are not altered in patients with normal albumin-creatinine ratio.

Higher urinary excretion of these markers is associated with microalbuminuria. Cherney et al. consider that these markers could have a role in assessing the risk of DN in patients with type 1 DM [94].

In type 1 DM patients, renal hyperfiltration is related to increased excretion of inflammatory cytokines/chemokines [95].

Tashiro et al. found in type 2 DM patients that IL8 is high in early stages of DN and MCP-1 increases in advanced stages [96].

A study on type 2 DM patients with normoalbuminuria and microalbuminuria found higher values of IL8, IP10, MCP-1, G-CSF, EOTAXIN, and RANTES in patients with microalbuminuria than in normoalbuminurics or in controls. Their assessment would be useful in the early diagnosis and treatment of DN [97].

Ibrahim and Rached also found that urinary MCP-1 is higher in patients with microalbuminuria than in normoalbuminurics or healthy controls [98].

3.3.2. Urinary Orosomucoid. Orosomucoid represents a glycoprotein involved in inflammatory processes.

Urinary orosomucoid has higher values in type 1 DM patients with normoalbuminuria than in controls. These values increase in patients with microalbuminuria and macroalbuminuria [99]. Type 2 DM patients presented increased excretion of orosomucoid in the urine, in parallel with the excretion of immunoglobulin G, ceruloplasmin, and transferrin [16]. El-Beblawy et al. appreciate that orosomucoid is a significant independent factor for diabetic microvascular complications and can be considered as an early marker of renal injury [100].

Urinary orosomucoid excretion rate in type 2 DM patients predicts cardiovascular mortality [101].

Urinary markers of inflammation are useful for assessing inflammatory processes in DN, even in early stages.

3.4. Oxidative Stress Biomarkers. Oxidative stress plays an important part in the development and progression of DN [102].

3.4.1. Urinary 8-Oxo-7,8-dihydro-2-deoxyguanosine (8-oHdG). 8-oHdG is produced secondary to oxidative DNA damage. It is eliminated into the urine without being metabolized [103]. At present, it is considered a marker for oxidative stress.

After a 5-year follow-up, Hinokio et al. find that 8-oxodG in urine is a useful clinical marker to predict the development of diabetic nephropathy in diabetic patients. There was a significant progression of diabetic nephropathy in the patients with higher excretion of 8-oxodG in urine compared with the patients with moderate or lower excretion of 8-oxodG [104].

Leinonen et al. reported increased excretion of 8-oHdG in type 1 DM patients 9 years after the onset of disease, mainly related to poor glycemic control [105].

The urinary 8-oHdG marker of oxidation would be, according to Broedbaek et al., a predictor of long-term mortality in DM [106].

3.4.2. Heart Fatty Acid Binding Protein (H-FABP). Heart fatty acid binding protein (H-FABP) is a marker of distal tubular damage.

In a study on a cohort of type 1 and type 2 DM patients and an assessment of their markers of glomerular lesions (IgG), markers of proximal tubular lesions (urinary KIM-1, NAG, NGAL, and cystatin), and a marker of distal tubular lesions (urinary H-FABP) in relationship with albuminuria and GFR, Nauta et al. reported higher values of urinary NAG, NGAL, and H-FABP in normoalbuminurics than in controls. On the other hand, the values of urinary cystatin C were low [107].

This shows that normoalbuminuric DM patients present both proximal and distal tubular lesions.

3.4.3. Urinary Advanced Glycation End Products (AGE). AGE eliminated in the urine induce a toxic tubular effect producing tubular dysfunction.

In type 2 DM patients with normoalbuminuria high values of urinary alpha-1-microglobulin and of urinary KIM-1 were found secondary to tubular dysfunction prior to the onset of microalbuminuria. At the same time, urinary AGE were high, being correlated with these markers [108].

Turk et al. found in type 2 DM patients high values of urinary AGE in 50% of the patients with normoalbuminuria and in 85% of those with microalbuminuria [109].

Pentosidine, a component of AGE, is a biomarker for their formation and accumulation [110].

Piarulli et al. found in patients with microalbuminuria higher values of pentosidine than in patients with normoalbuminuria [111].

3.4.4. Podocytes. Podocyte lesions appear during DM and DN, respectively, the disease being considered a podocytopathy as mentioned above.

The assessment of podocyte injury can be accomplished by monitoring the number of podocyte cells in the urine or, more precisely, by means of using podocyte urinary biomarkers (podocalyxin and nephrine).

A study on DM patients found that the values of the number of urinary podocytes in normoalbuminuric patients are not significantly different from those of controls. In patients with microalbuminuria and nephrotic syndrome, the number of urinary podocytes is higher. It is correlated with urinary osteopontin and urinary IgM [33].

Urinary podocalyxin originates in the podocyte apical surface, occurring in vesicle form. In DM patients, the podocalyxin level presented higher levels in patients with microalbuminuria than in patients with normoalbuminuria [112].

Another study on DM patients found high values of urinary podocalyxin in more than half of the patients with normoalbuminuria, these values being higher in patients with microalbuminuria and macroalbuminuria.

Urinary podocalyxin is correlated with the values of urinary NAG and of urinary beta 2 microglobulin [113].

Hara et al. consider that urinary podocalyxin can be an early biomarker for detecting early podocyte injury in patients with DM.

Zheng assessed the urinary microRNA profile of podocyte-associated molecules (synaptopodin, podocalyxin, CD2-AP, α-actin4, and podocin) as biomarkers in patients with normoalbuminuria, microalbuminuria, and macroalbuminuria and they reported its increase during the progression of DN [114].

3.4.5. Vascular Endothelial Growth Factor (VEGF). VEGF is a proangiogenic factor produced mainly by the podocytes at nephron level. Urinary VEGF can be considered a podocyte biomarker.

Urinary VEGF was detected in type 2 DM patients, being correlated in these patients with urinary alpha-1-microglobulin, a biomarker for proximal tubular lesions [62].

Kim et al. found that VEGF is excreted at higher values than controls in normoalbuminuric type 2 DM patients. The values increase in patients with microalbuminuria and macroalbuminuria [115].

Fetuin A is glycosylated glycoprotein was considered an inhibitor for ectopic calcium deposition and promoter of insulin resistance. Fetuin A inhibits the calcification of atherosclerotic plaques in diabetes mellitus [116]. It was found that elevated urinary Fetuin A excretion is a risk for development of diabetic nephropathy [117].

3.5. Other Urinary Biomarkers Used in Evaluating Early DN. Numerous urinary markers have been suggested for assessing early DN. Some of them have been introduced in use only recently.

Urinary retinol-binding protein is a low molecular weight protein that was found to have high urinary values (together with NAG) in normoalbuminuric patients, reflecting tubular dysfunction in early DN [118].

The value of serum retinol-binding protein 4 as a biomarker in assessing the severity of coronary artery disease is to be mentioned [119].

Urinary retinol-binding protein 4 as a biomarker in assessing DN needs further studies.

Urinary vitamin D binding protein can plays the role as biomarker. In type 2 DM it is attributed a potential role in early diagnosis of DN [120].

Urinary heme oxygenase-1 was found in type 2 DM patients before the onset of significant albuminuria, thus being a possible biomarker of early DN [121]. In fact oxidative stress activation is expected in DN.

Periostin is a cell adhesion molecule which is not normally present in kidneys. In tubulointerstitial lesions it is however expressed in the kidneys, being eliminated in the urine. This is why urinary periostin could be used as a marker of injury at this level.

Since high levels of periostin can be identified in DM patients before significant albuminuria, periostin could represent a marker of diabetic renal injury [122].

Urinary alpha klotho presents higher values in normoalbuminuric type 2 DM patients than in controls. It can also be a marker of diabetic injury [123].

Analyzing a group of normoalbuminuric, microalbuminuric, and macroalbuminuric type 2 DM patients, Sun et al. noted that the urinary level of microvesicle-bound dipeptidyl peptidase-IV is related to the severity of DN [124].

Recent studies point to the usage of urine-specific microRNA as a biomarker for early stages of DN. Analyzing the studies in the literature, Yang et al. issued the hypothesis that urine-specific microRNA would be a marker that can be used in the early stages of DN [125].

Recently, Argyropoulos highlighted the predictive role of urinary microRNA regarding microalbuminuria in type 1 DM [126].

Adipokine zinc-alpha-2 glycoprotein is assigned to the major histocompatibility complex class I of proteins [127].

Urinary adipokine zinc-alpha-2 glycoprotein is present earlier than microalbuminuria in diabetic nephropathy. It could be a useful biomarker for diagnosing early DN [128]. Lim et al. also appreciate adipokine zinc-alpha-2 glycoprotein as a novel urinary biomarker for normoalbuminuric diabetic nephropathy [129].

3.5.1. Proteomics. At present proteomic investigations are engaged in identification of new urinary biomarkers to be used in the early diagnosis of DN.

In fact, proteomics studies noted the fact that microalbuminuria is not a perfect biomarker for early detection of DN [130, 131].

Urinary proteomics begins to stand out as a noninvasive method of detecting early DN.

Among proteomics studies on diagnosing DN we can mention those of Zürbig et al., who reported that collagen fragments were a prominent biomarker 3–5 years before the onset of microalbuminuria [132].

A potential role is also attributed to exosome proteomics for identifying new biomarkers for DN [133]. Zubiri et al. showed a panel of 3 proteins which is differentially present in urinary exosomes from DN patients [134]. Urinary proteomic analysis can have an important role in the implementation of new biomarkers in DN [135].

At present, the prospect of discovering new biomarkers in DM and DN respectively is incumbent both on proteomics and on genomics, transcriptomics, and metabolomics [136].

4. Conclusions

Urinary biomarkers allow an assessment of early DN.

Microalbuminuria, although frequently contested as a biomarker of early DN, is used so far as reference biomarker in assessing other urinary biomarkers in early DN. Until present there is no other biomarker that can substitute in practice microalbuminuria, the new biomarkers being sustained by limited studies and requiring validation.

The concomitant assessment of several urinary biomarkers in relationship with microalbuminuria could represent at present a method of diagnosing early DN. The great progress in discovering new biomarkers could lead to the development of an "ideal" urinary biomarker to detect early diabetic DN in the future.

Progresses in the field of urinary biomarkers in DN, promising both in proteomics and in other modern techniques, develop remarkably at present.

Disclosure

The supporting source had no involvement in study design, in collection, analysis, and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Cristina Gluhovschi and Gheorghe Gluhovschi contributed equally to this paper.

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Review Article

Histone Acetylation and Its Modifiers in the Pathogenesis of Diabetic Nephropathy

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Diabetic nephropathy (DN) remains a leading cause of mortality worldwide despite advances in its prevention and management. A comprehensive understanding of factors contributing to DN is required to develop more effective therapeutic options. It is becoming more evident that histone acetylation (HAc), as one of the epigenetic mechanisms, is thought to be associated with the etiology of diabetic vascular complications such as diabetic retinopathy (DR), diabetic cardiomyopathy (DCM), and DN. Histone acetylases (HATs) and histone deacetylases (HDACs) are the well-known regulators of reversible acetylation in the amino-terminal domains of histone and nonhistone proteins. In DN, however, the roles of histone acetylation (HAc) and these enzymes are still controversial. Some new evidence has revealed that HATs and HDACs inhibitors are renoprotective in cellular and animal models of DN, while, on the other hand, upregulation of HAc has been implicated in the pathogenesis of DN. In this review, we focus on the recent advances on the roles of HAc and their covalent enzymes in the development and progression of DN in certain cellular processes including fibrosis, inflammation, hypertrophy, and oxidative stress and discuss how targeting these enzymes and their inhibitors can ultimately lead to the therapeutic approaches for treating DN.

1. Introduction

DN is one of the most serious diabetic microvascular complications and the leading cause of end-stage renal diseases (ESRD); it brings about heavy social and economic burden worldwide, particularly in the developed countries. Both type 1 and type 2 diabetic patients presented indistinguishable and variable pathological changes and clinical course; the prognosis is difficult to predict because of diverse pathogenesis. Clinically, DN is characterised by different degrees of proteinuria, albuminuria, increased serum creatinine (Scr), decreased glomerular filtration rate (GFR), and ESRD [1, 2]. Importantly, DN also increases the risks for the development of diabetic macrovascular complications including heart attacks and strokes [3, 4]. Pathologically, DN associated histological structural changes include glomerular mesangial expansion, glomerular basement membrane (GBM) thickening, glomerular sclerosis known as Kimmelstiel-Wilson lesions caused by excessive extracellular matrix (ECM) proteins accumulations, and tubulointerstitial fibrosis in the advanced stages [1, 5]. Arterial hyalinosis of the afferent and efferent arterioles is often prevalently caused by endothelial dysfunction and inflammation [2, 6, 7], which will lead to glomerular hyperfiltration.

In the development and progression of DN, resident kidney cells are affected by hyperglycemia: including mesangial cells, podocytes, endothelial cells, smooth muscle cells, inflammatory cells, myofibroblasts, and cells of tubular and collecting duct system [4]. Multiple contributors including environmental and genetic factors are associated with the pathogenesis of DN, which cause metabolic, hemodynamic, and biochemical changes in the diabetic kidneys [8]. Main pathways leading to DN include intracellular PKC activation and increased polyol pathway flux, production of reactive oxygen species (ROS) and advanced glycation end products (AGEs), and hypertension and glomerular hyperfiltration leading to shear stress and mechanical stretch [8, 9]. Increased blood glucose activates the renin-angiotensin system (RAS), TGF-β-Smad-MAPK pathway, JAK-STAT pathway, and G-protein signaling [7]; aberrant expression of

ECM proteins and deregulated expression of cyclin kinases and their inhibitors; transcription factor such as NF-κB [10], proinflammatory cytokines like TNF and IL-1 [11], and toll-like receptors 4 (TLR4) [12], which are considered to exert hemodynamic, proinflammatory, and profibrotic effects on kidney cells [8, 13]. There is cross-talk among the above-mentioned signaling pathways, which can amplify aberrant pathogenetic genes expression and lead to the progression of DN. In addition, the phenomenon of metabolic memory regulated by epigenetic mechanisms can promote these genes expressions [14, 15]. Although a lot of biochemical and molecular mechanisms and pathways have been broadly studied in the pathogenesis of DN, the undeniable fact is that the progressive incidence and prevalence of DN worldwide still exist, suggesting that more investigations will be needed in the future.

Emerging evidences suggest that multiple signaling pathways activations and key transcription factors (TFs) are associated with the pathophysiology of DN, which could be influenced by epigenetically regulated mechanisms in chromatin (histones form a complex structure with DNA), including DNA methylation, posttranslational modifications (PTMs), and noncoding RNAs (ncRNA), which can modulate gene expression in the cell-type-specific pattern. Core histones are subject to diverse PTMs including histone lysine acetylation (HKAc), histone lysine methylation (HKme), phosphorylation, ubiquitination, and sumoylation. We have implicated the roles of HKme in the pathogenesis of DN [7], especially in the metabolic memory phenomenon pertinent to DN [2, 16, 17]. Global acetylation alterations have been seen in a lot of human diseases including cancer and nervous system diseases [18], whereas the roles of HAc in the pathogenesis of DN are rarely mentioned.

Recently, some studies showed that HAc level is linked to DN, HATs and HDACs also participate in the pathogenesis of DN, and the research regarding HAc and the covalent enzymes is not enough to yield a clear picture about DN so far. In this review, we describe some progress associated with the molecular mechanism underlying DN, with specific emphasis on HAc and acetylation on nonhistone proteins as important regulators of gene expression in renal cell under diabetic conditions; the regulators of HAc such as HATs as well as HDACs in the development and progression of DN; the inhibitors of HATs/HDACs in the DN pathogenesis and their therapeutic potentials for DN.

2. Histone and Nonhistone Acetylation in DN

Reversible acetylation of histones has been demonstrated for more than 50 years [19]. Dynamic balance of histone acetylation and deacetylation can regulate gene expression, chromosome assembly, mitosis, and PTMs [20], by altering the chromatin structure and the accessibility to TFs without affecting the sequence of DNA [21]. HAc is highly reversible and dynamic, which can be catalyzed by HATs or HDACs, respectively. HAc on H3 and H4 has been considered as marker of an "open" configuration of chromatin. HKAc at N-terminal tails can facilitate gene transcription through neutralizing the positive charge of histone residues and weakening the binding of histone to negatively charged DNA [22, 23]. HKAc, such as H3K9Ac, H3K14Ac, and H4KAc, is generally linked to permissive gene expression [24], while histone deacetylation is often associated with chromatin condensation and gene transcriptional repression [25, 26].

Several previous studies have shown that HKAc at the insulin gene promoter was specific to β cells and islet-derived precursor cells, which was highly correlated with the recruitment of p300 [27, 28]. *In vitro* studies with HDAC inhibitors (HDACI) suggested that HKAc was essential in the development of pancreas [29]. These findings cannot fully demonstrate the underlying mechanism of DN; in this review, we will discuss the current opinions of HAc and nonhistone acetylation on inflammation, fibrosis, and oxidative stress in the development and progression of DN (Table 1).

Recent studies have demonstrated that dysregulated acetylation of core histone is associated with DN. Diabetic patients showed that levels of H3 acetylation at lysine 9 and 14 and H4 acetylation at lysine 5, 8, and 12 were increased at TNF-α and COX-2 inflammatory genes promoters in human blood monocytes [30]. Another study showed that oxidized lipids could increase H3K9/14Ac at MCP-1 and IL-6 gene promoters in a CREB/p300-dependent manner, along with the inflammatory genes expression [31]. Advanced DN in db/db mice underwent by uninephrectomy is specifically associated with increased acetylation of H3K9 and H3K23 [32]. A recent study revealed that acetylation of H3K9, H3K18, and H3K23 were significantly increased in the renal cortex of Akita mice, HG and NaB-induced H3K9 and H3K18 acetylation was elevated in the mesangial cells also, which were associated with inflammatory factors such as MCP-1, ICAM-1, VCAM-1, and iNOS expression linked to the development of DN [33]. HKAc mediated by HATs can increase transcriptional activity of proinflammatory NF-κB under diabetic conditions [30]. Thioredoxin-interacting protein (TXNIP) has been demonstrated to play an important role in the pathogenesis of DN. HG-induced TXNIP expression was associated with the stimulation of activating H3K9Ac in MCs of diverse species, which could drive the expression of proinflammatory genes predisposing to DN [34].

TGF-β1 is established to be involved in the pathogenesis of DN, the underlying mechanism of which is still unclear. TGF-β1 treatment could increase acetylation of histone (H3K9, H3K14, and H3K27) as well as Ets-1 in mouse renal glomerular mesangial cells; furthermore, acetylation of Ets-1 and histone H3 was increased in glomeruli from diabetic db/db mice also, both of which can increase *miR-192* expression contributing to DN [35]. TGF-β1 can also mediate the effects of HG [2]. TGF-β1 treatment increased H3K9/14Ac at the PAI-1 and p21 promoters near Smad and SP1 binding sites in RMCs, acetylation of Smads was also increased [36, 37], and HG-treated RMCs exhibited increased levels of H3K9/14Ac that can be blocked by TGF-β1 antibodies, which played an important role in TGF-β1 and HG-induced deregulated gene expression associated with hypertrophy and fibrosis linked to DN [2]. HG stimulation can also increase H3K9/14Ac at the RAGE, PAI-1, and MCP-1

Table 1: Reported histone lysine and nonhistone acetylation in DN.

Ac proteins	Acetylation site	Target genes	Target renal loci	Effects in DN	References
Histone lysine	H3K9	MCP-1, ICAM-1, andVCAM-1; TXNIP	db/db mice kidney; Akita mice renal cortex, MCs	Advanced diabetic glomerulosclerosis; inflammation	[32–34]
	H3K9/14	TNF-α, COX-2; MCP-1, IL-6; PAI-1, p21; RAGE; CTGF, FN	Human blood monocytes; rat VSMCs; MMCs, db/db mice glomeruli; RMCs; STZ-induced mice	Inflammation; increased *miR-192* expression; hypertrophy, fibrosis	[30, 31, 35, 36, 38, 39]
	H3K18	MCP-1, ICAM-1, and VCAM-1	Akita mice renal cortex, MCs	Advanced diabetic glomerulosclerosis; inflammation	[33]
	H3K23		db/db mice kidney	Advanced diabetic glomerulosclerosis	[32, 33]
	H3K27		MMCs, db/db mice glomeruli	Increased *miR-192* expression	[35]
	H4	GRP78, CHOP	STZ-induced rat kidney	Cell apoptosis, proteinuria, and increase of Scr	[44]
	H4K5/8/12	TNF-α, COX-2	Human blood monocytes	Inflammation	[30]
Nonhistone proteins	Ets-1		MMCs, db/db mice glomeruli	Increased *miR-192* expression	[35]
	Foxo4	*Bcl2l11*	Podocyte	Promoting apoptosis	[45]
	NF-κB	TGF-β1, FN, and type IV collagen	RMCs, diabetic rats	UAE increase, matrix expansion, and ECM deposition	[46]
	NF-κB p65	MCP-1, PAI-1, and TGF-β1	Mice and human diabetic kidneys, human podocytes; RMCs;	Kidney injury; inflammation;	[47, 48]
	STAT3		mice and human diabetic kidneys; human podocytes	Kidney injury	[47]
	Smad3	SREBP-1; type IV collagen	MMCs; iHMCs	Inducing glomerulosclerosis, increased albuminuria	[49, 50]
	Nephrin	WT-1, TGF-β1, and FN	STZ-induced FVB mice, podocyte	Ameliorate HG-induced podocyte dysfunction	[51]

promoters, which can be further augmented by HG+Ang II (HG/A), suggesting the key roles of H3K9/14Ac in the key DN-related genes expression [38]. Excessive H3K9/14Ac levels were reported at the CTGF, PAI-1, and FN-1 promoters in diabetic kidneys, which were associated with p300/CBP activation [39]. Although there is a conflicting result in an animal study that the level of H3K9/14Ac was decreased in the STZ-induced type 1 diabetic rat kidney [40, 41], the majority of HAc is involved in the development and progression of DN.

For the past few years, the phenomenon "metabolic memory" has been implicated in the pathogenesis of diabetes and its complications such as DN. A study of patients from DCCT conventional treatment groups showed that there was association between HbA1c level and H3K9Ac; hyperacetylated promoters included more than 15 genes related to the NF-κB pathway and could be enriched in genes associated with diabetic complications [42], which may be a possible epigenetic explanation along with HKme [16, 17, 43] for metabolic memory phenomenon in humans.

Endoplasmic reticulum stress (ERS) is an important mechanism responsible for the pathogenesis of DN. Histone H4 acetylation levels are increased at glucose-regulated protein (GRP78) promoters and decreased at C/EBP-homologous protein (CHOP) promoters, which are associated with renal cell apoptosis, proteinuria, and increases of Scr; these results provide initial experimental evidences for understanding the mechanism of DN [44].

Apart from HAc, nonhistone proteins acetylation can also take part in the pathogenesis of DN. Fork box O4 (Foxo4) transcription factor can be activated to promote podocyte apoptosis by AGEs through *Bcl2l11* expression, at the same time, AGE-BSA can also increase Foxo4 acetylation; a recent study showed that alteration of Foxo4 acetylation and downregulation of Sirt1 expression in DM promote podocyte apoptosis; Foxo4 acetylation reduction could be a therapeutic potential for preventing diabetic podocyte loss [45]. Enhanced NF-κB acetylation level was present in both diabetic rats and HG-treated RMC leading to DN in another study, which can be dampened by 3,5-diiodothyronine (T2) involved regulation of SIRT1 [46]; acetylation of NF-κB p65 and STAT3 was increased in both mice and human diabetic kidneys and AGEs induced human podocytes, suggesting their critical roles in DN [47]. NF-κB p65 acetylation was also increased by HG in RMCs, PNS could protect diabetic kidney through decreasing induction of inflammatory cytokines and TGF-β1 [48]. Smad 3 acetylation has been implicated in the pathogenesis of DN recently [49, 50], overexpression of transcription factor SREBP-1 induces glomerulosclerosis of DN; SREBP-1a K333 acetylation by CBP is required for Smad3 association and SREBP-1 transcriptional activity; both Smad3 and SREBP-1a activation regulates TGF-β1 transcriptional responses associated with DN, SREBP-1 inhibition could be a novel therapeutic strategy for DN [49]. Nephrin acetylation in diabetic podocytopathy has seldom been addressed before, a recent study showed that nephrin acetylation was reduced in STZ-induced diabetic mice kidney; increasing miR-29a may protect diabetic podocytopathy by modulating nephrin acetylation [51].

3. HATs and DN

There are two groups of HATs based on their cellular localizations. Type A HATs (nuclear) exist in nucleus, including (1) GNAT (GCN5) family such as GCN5, p/CAF, and ELP3, (2) MYST (HMOF/MYST1, HBO1/MYST2, MOZ/MYST3, MORF/MYST4, and TIP60) family, (3) p300/CBP, (4) basal TF family (TFIIIC and TAF1), and (5) NRCF family, SRC, and ACTR/NCOA3 [18], which can acetylate nucleosomal histones and other chromatin-associated proteins, while type B HATs are cytoplasmic and acetylate newly synthesized histones [52]. HKAc is generally mediated by HATs including p300, CBP, p/CAF, and TIP60, which is associated with gene activation via adding acetyl groups. In addition, HATs can also regulate gene expression through acetylation of nonhistone proteins such as Smads, p53, SP1, and NF-κB.

Among the studies of HATs and their links with DN development, *in vitro* and *in vivo* studies showed that HATs CBP and p/CAF recruitment was increased under diabetic conditions, which led to upregulated HKAc at inflammatory genes promoters continent with the gene expression [30, 53]. It was implicated that p300 played important roles in oxidative stress-induced PARP and NF-κB signaling in HG-treated endothelial cells and diabetic kidneys [53–55]; further study showed that HG upregulated p300, which increased HAc at promoters of key ECM protein FN, as well as vasoactive factors such as ET-1 and VEGF in endothelial cells [56]. Another study showed that TGF-β1 increased H3K9/14Ac by recruiting the HATs p300 and CBP; TGF-β1 treatment also increased association of p300 with Smad2/3 and SP1, cotransfection experiments showed that p300 and CBP, but not p/CAF, upregulated transcriptional activity of PAI-1 and p21 promoters and increased TGF-β1-induced gene expression. On the contrary, inhibition of CBP and p300 by overexpressing dominant-negative mutants could block TGF-β1-induced gene expression [36]. P/CAF was found sharply increased in the renal cortex of Akita mice, while GCN5 was significantly decreased in the HG group, suggesting that the inflammatory genes expressions were related to DN [33]. *In vivo* and *in vitro* results of another report showed that p/CAF was closely related to H3K18Ac levels at inflammatory molecules ICAM-1 and MCP-1 promoters, which could be a potential therapeutic agent for inflammation-related renal diseases including DN [57]. All the data implied that HATs have critical roles in acetylating both histones and nonhistone proteins in the pathogenesis of DN; these results point to the necessity of further studies on the HATs activity in the development of DN, which may be therapeutic targets in the future.

4. HATs Inhibitors and DN

In preclinical trials, small-molecule HATs inhibitors have been shown to sensitize cancer cells to ionizing irradiation [58]. Curcumin, the p300/CBP inhibitor [59], extracted from rhizomes of turmeric *Curcuma longa* [60], which was supposed to be a new target molecule for treating CNS disorders and cancer [61, 62], was firstly reported to prevent the development of DN involved in the changes of PTMs of

histone H3 including acetylation and phosphorylation and the changes in HSP-27 and p38 expression in diabetic rats [40]. Curcumin could also prevent HG-induced key ECM genes and vasoactive factors (eNOS and ET-1) expression levels associated with DN in endothelial cells [56]; it was able to reverse the upregulation of vasoactive factors, TGF-β1 and ECM protein FN in STZ-induced diabetic kidneys, which was associated with p300 and NF-κB activity changes [63]. Curcumin was also found to reverse HG-induced cytokines (IL-6, TNF-α, and MCP-1) production in human monocytes via epigenetic changes involving NF-κB [64], but dietary curcumin failed to decrease albuminuria either before or after diabetes induction [65]. Curcumin analogue, C66, has been demonstrated to significantly and persistently prevent renal injury and dysfunction in diabetic mice via downregulation of JNK activation and consequent suppression of diabetes-related increases in p300/CBP expression and histone acetylation (H3K9/14Ac) [39].

In a recent study, C646, a novel p300/CBP specific inhibitor, has been declared to specifically suppress the growth of CBP-deficient hematopoietic and lung cancer cells *in vivo* and *in vitro* [66]. In another *in vitro* study, histone H3Ac activated TGF-β1/Smad3 pathway during EMT of human peritoneal mesothelial cells; C646 could reverse the mesenchymal phenotype transition [67]. C646 was also reported reversing acetylation involved in HG-induced TXNIP expression leading to DN [34].

5. HDACs and DN

To date, 18 HDACs have been identified in humans and divided into 4 distinct classes based on their homology to yeast HDAC, in which Class I (HDAC1, 2, 3, and 8), Class II including IIa (HDAC4, 5, 7, and 9) and IIb (HDAC6 and 10), and Class IV (HDAC11) have structurally similar zinc-dependent active sites, whereas Class III, sirtuins (SIRTs1-7), are zinc-independent but require cofactor nicotinamide adenine dinucleotide (NAD) [52]. HDACs can remove acetyl groups from conserved lysine residues and nonhistone proteins and generally act as corepressors with some exceptions [2]. Evidence for mechanisms by which HDACs act in controlling DN is accumulating. Most research related to the epigenetics of DN has focused on HAc; different classes of HDACs are involved in distinct pathways that engaged in the pathogenesis of DN.

Overexpression of HDAC1 and HDAC5 blocked TGF-β1-induced gene expression, whereas inhibition of HDACs upregulated H3K9/14ac and gene expression, further supporting the key inhibitory roles of HDACs in TGF-β1-induced gene expression [36]. A recent study showed that HDAC1 was significantly decreased in the renal cortex of Akita mice, while the levels of HDAC2 in Akita and WT mice were unchanged, and HDAC1 was significantly decreased in HG-cultured HBZY-1 cell, which can upregulate diabetes-, HG-, and NaB-induced histone hyperacetylation leading to inflammatory factors elevation associated with DN [33].

Glomerular sclerosis is also a core characteristic of DN resulting from excessive ECM deposition in the glomerular mesangium and the loss of glomerular epithelial cells, followed by aberrant fibrosis in the glomerular structure. HDAC2 activity was markedly increased in the kidneys of type 1 and type 2 murine models and TGF-β1 treated NRK52-E cells, which played an important role in the development of DN [68]. Knockdown of HDAC2 in cell culture reduced ECM components accumulation, further implicating the role of HDAC2 in the fibrosis. Oxidative stress is also of the view to play an important role in regulating fibrosis in DN [69]; a potent oxidative stress inducer H_2O_2 can increase HDAC2 levels [68], which may be an underlying mechanism in the pathogenesis of DN.

HDAC4 is regarded as a contributor to podocyte injury in type 1 and type 2 diabetic models and diabetic patients and could suppress autophagy related with podocyte injury in DN by deacetylating STAT1, suggesting that HDAC4 is important to accelerate DN in epigenetic and nonepigenetic mechanisms [70, 71].

SIRTs have been shown to be involved in diverse cellular processes such as insulin secretion, cell cycle, and apoptosis [72]. Dysfunction of SIRT1 may contribute to abnormal cancer metabolism, cancer stemness, neurological disorders, obesity, and diabetes [72]. A previous study showed that decreased SIRT1 level in diabetic kidney and intermittent fasting (IF) prevents this decrease; SIRT1-dependent deacetylation is thought to mediate p53 expression and activation, which could play a renoprotective effect of IF in diabetes [73]. Another report showed that resveratrol could prevent decreased SIRT1 and increased p53 expression in diabetic kidney, which could be responsible for preventing apoptosis in type 1 diabetic kidney [74]. Resveratrol has also been demonstrated to reduce oxidative stress and maintain mitochondrial function related with SIRT1 activation in HG-treated MCs and *db/db* diabetic mice [75, 76]. SIRT1 in proximal tubules (PT) has been reported to attenuate diabetic albuminuria by suppressing the overexpression of tight junction protein Claudin-1 via hypermethylation of the Claudin-1 gene in podocytes [77, 78]. Another previous report showed that SIRT1 could inhibit TGF-β1-induced glomerular mesangial cell apoptosis via Smad7 deacetylation [79], and overexpression of SIRT1 attenuated ROS-induced apoptosis in mesangial cells through p53 deacetylation and provided a new therapeutic strategy for kidney glomerular diseases [80]; TSG has been proven to protect DN through inhibiting TGF-β1 expression partially mediated by SIRT1 activation [81]. Conditional SIRT1 deletion in podocytes of diabetic db/db mice developed more acetylation of NF-κB p65 and STAT3, proteinuria, and kidney injury compared with db/db mice without SIRT1 deletion, suggesting the protective roles of SIRT1 in TFs acetylation on DN [47]. Dietary restriction was reported to ameliorate DN through regulation of the autophagy via restoration of SIRT1 in diabetic *fa/fa* rats [82]. The beneficial effects of SIRT1 on AGE-associated DN correlate with the activation of *Nrf2/ARE* antioxidative pathway [83, 84]. All the findings suggested the possibility of SIRT1 as the target of treatment in DN [85–87].

Taken together, these studies highlight important and different roles of HDACs in the pathways, and most of them are beneficial, suggesting HDACs will be the targets for

Table 2: Reported HATs/HDACs in DN.

Enzyme category	Enzymes	Catalyzed site	Target renal loci	Effects in DN	References
HATs	CBP	H3K9/14, H4K5/8/12	Human monocytes; RMCs	Inflammation; increased TGF-β1-induced genes expression	[30, 36]
	GCN5		Akita mice renal cortex	Inflammation	[33]
	P300	H3K9/14	Endothelial cell, diabetic rats; RMCs	Inflammation, FN, vasoactive factors; increased TGF-β1-induced genes expression	[36, 54, 56]
	p/CAF	H3K9/14, H4K5/8/12; H3K18	Human monocytes; Akita mice renal cortex; db/db mice, human renal proximal tubule epithelial cell line	Inflammation	[30, 57]
HDACs	HDAC1	H3K9/14; H3K9, H3K18	RMCs; Akita mice, HBZY-1 cell	Blocking TGF-β1-induced gene expression; affecting inflammatory factors	[33, 36]
	HDAC2	H3/H4	Type1/2 murine models, NRK52-E cells	Promoting fibrosis	[68]
	HDAC4		Db/db mice, STZ-induced rats, diabetic patients	Contributing to podocyte injury	[70]
	HDAC5	H3K9/14	RMCs	Blocking TGF-β1-induced gene expression	[36]
	SIRT1	NF-κB, STAT3	Renal tubular cells, podocyte; GMCs; db/db mice; diabetic *fa/fa* rats	Attenuating albuminuria; inhibiting cell apoptosis; attenuating kidney injury; regulating autophagy; reducing oxidative stress	[47, 75–80, 82–84]

the prevention of DN despite the fact that further studies are needed.

6. HDACIs and DN

The present HDACIs include both natural and synthetic compounds and are subdivided into 5 categories: short-chain fatty acids, cyclic peptides, benzamides, electrophilic ketones, and small-molecule hydroxamic-acid-derived compounds [52, 88]. HDACIs are regarded as potential anticancer agents and are promising for the treatment of a lot of diseases such as inflammation and neurological diseases [72]. Recently, HDACIs have been identified as a novel class of potential therapeutic agents for DN [89]. Here we list some progress of HDACIs applied in the treatment of DN regarding antifibrotic, anti-inflammatory, and antioxidative effects.

Nevertheless, most of the HDACIs are nonselective and target both nuclear histones and cytoplasmic nonhistone proteins [23]. It was found that millimolar concentrations of *n*-butyrate induce accumulations of acetylated histones in cells in the 1970s and inhibited deacetylation [72, 90, 91]. Sodium butyrate (NaB, a nonselective inhibitor of HDACs), a short-chain fatty acid, can upregulate HAc levels, promote tumor cell senescence and apoptosis, and inhibit tumor cell proliferation [20]. NaB was used as animal feed additive and played a major role in the treatment of neurodegenerative conditions. *In vivo*, it was reported that NaB could not only decrease blood glucose, creatinine, and urea but also ameliorate histological changes, fibrosis, apoptosis, and DNA damage in the kidneys of juvenile diabetic rats [92]. Further studies are needed to provide more evidences and theoretical basis in treating DN.

SAHA (suberoylanilide hydroxamic acid, vorinostat), a nonselective HDACI, designed and synthesized as a hybrid polar compound that can strongly induce erythroid differentiation [72, 93], is orally bioavailable and clinically applicable. SAHA can reduce albuminuria, glomerular hypertrophy, and glomerular type IV collagen deposition through an eNOS-dependent mechanism, without affecting blood pressure or blood glucose concentration [94]. Indeed, another study showed that SAHA attenuated early renal enlargement in STZ-induced diabetic rats, which is supposed to be mediated partly through downregulating EGFR [95]. These results indicated the key role of SAHA in attenuating fibrosis and oxidative damage in DN.

Trichostatin A (TSA), the natural product isolated from a *Streptomyces* strain, originally identified as an antifungal antibiotic, was discovered to have potent HDAC inhibition activity in 1990 [72]. TSA was reported to act as an agent in preventing DN in diabetic rats [32], by blocking TGF-β1-induced ECM accumulation [68] and EMT in diabetic kidneys [68] as well as in renal epithelial cells [96]; knockdown of HDAC2 had similar effect of TSA treatment mediated by ROS.

Valproic acid (VPA), a broad-spectrum HDACI, is a first-line drug used for the treatment of epilepsy and migraine. VPA treatment alleviated renal injury and fibrosis in STZ-induced diabetic kidney by preventing myofibroblast activation and fibrogenesis through HDAC4/5/7 inhibition in

Table 3: Effect of inhibitors of HATs/HDACs in DN.

Inhibitors category	Name	Target genes	Target renal loci	Effects in DN	References
HATs inhibitors	Curcumin	ECM genes, vasoactive factors; inflammatory genes;	STZ-induced rats; endothelial cell; human monocytes	Reversing ECM proteins and vasoactive factors upregulation; reverse HG-induced cytokines	[40, 56, 63, 64]
	C66	CTGF, PAI-1, and FN-1	STZ-induced mice	Preventing renal fibrosis and dysfunction	[39]
	C646	TXNIP	Diabetic Sur1-E1506K(+/+) mice	Reversing acetylation leading to DN	[34]
HDACs inhibitors	NaB		Juvenile diabetic rats	Decreasing blood glucose, creatinine, and urea; ameliorating histological changes, fibrosis, and apoptosis	[92]
	SAHA	type IV collagen	STZ-induced mice, HUVECs; STZ-induced rats, NRK	Decreasing albuminuria, glomerular hypertrophy	[94, 95]
	TSA		STZ-induced rats, NRK52-E	Blocking TGF-β1 induced ECM accumulation and EMT	[68, 96]
	VPA	TGF-β1, CTGF, FN, collagen I, COX-2, and ICAM-1	STZ-induced diabetic rats	Alleviating renal injury and fibrosis; ameliorating podocyte and renal injury	[97, 98]

a dose-dependent manner [97], VPA has also been proven to ameliorate the podocyte and renal injuries by facilitating autophagy and inactivation of NF-κB/iNOS pathway [98]. A recent study showed that VPA can attenuate renal injury in a rat model of DN, by upregulating the histone H4 acetylation levels at the promoter of GRP78 and downregulating the histone H4 acetylation at the promoter of CHOP [44].

To our knowledge, at the time of the present review, the molecular implications of HDACIs were identified in the treatment of DN, and the development of selective HDACIs in preventing DN may be part of the most prevalent areas in the drug discovery.

7. Conclusions and Perspectives

Recent research has concentrated on histone modifications to provide a reliable theoretical basis for clinical treatment. A comprehensive understanding of HAc mechanisms can give rise of novel therapeutic options for DN. Increasing *in vitro* and *in vivo* evidences implicated that reversible histone and nonhistone acetylation play important roles in the pathogenesis of DN, suggesting that HAc regulation could be promising therapeutic targets for DN. HATs and a small number of HDACs provide a central mechanism for regulating gene expression and cellular signaling events in DN (Table 2). Experimental evidences suggest that HATs/HDACs inhibitors and a large number of HDACs can delay the development and progression of DN (Tables 2 and 3). HATs inhibitor curcumin and its analogue C66 could protect renal injuries in diabetic patients and diabetic animal models; Apelin-13 and Esculetin treatment could be innovative therapeutic agents for DN via regulation of HAc also [33, 40, 41]. Continued research is needed to better understand the roles of HAc in the process of DN, the modifiers and the mechanism that regulate them, and address the curative potential of more selective HATs inhibitors and HDACI in treating DN.

Competing Interests

The authors do not have any conflict of interests to declare.

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Review Article

Silymarin in Type 2 Diabetes Mellitus: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

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Type 2 diabetes mellitus (T2DM) is associated with increased risk of cardiovascular disease and nephropathy—now the leading cause of end-stage renal disease and dialysis in Europe and the United States. Inflammation and oxidative stress play a pivotal role in the development of diabetic complications. Silymarin, an herbal drug with antioxidant and anti-inflammatory properties, may improve glycemic control and prevent the progression of the complications. In a systematic review and meta-analysis including five randomized controlled trials and 270 patients, routine silymarin administration determines a significant reduction in fasting blood glucose levels (−26.86 mg/dL; 95% CI −35.42–18.30) and HbA1c levels (−1.07; 95% CI −1.73–0.40) and has no effect on lipid profile. Benefits for silymarin on proteinuria and CKD progressions are reported in only one small study and are uncertain. However, being aware of the low quality of the available evidence and elevated heterogeneity of these studies, no recommendation can be made and further studies are needed.

1. Introduction

Type 2 diabetes mellitus (T2DM) is one of the fastest growing health problems in the world, reaching epidemic proportion; globally it is estimated that 382 million people suffer from diabetes, that is, a prevalence of 8.3% [1]. T2DM is the fourth leading cause of death in developed countries, with a twofold excess mortality and twofold to fourfold increased risk of coronary heart disease and stroke [2]. About 20–30% of patients with diabetes develop evidence of nephropathy, now the leading cause of end-stage renal disease (ESRD) and dialysis in the US and in Europe [3]. Importantly, diabetes places large financial demands on the healthcare system: an estimated $245 billion in 2012 in the US, which is expected to rise with the increasing number of newly diagnosed individuals [4].

Managing diabetes is a considerable challenge to patients, providers, and healthcare systems all over the world. Better treatment options to reduce both the development and progression of diabetes complications are urgently required. In this context, it is important to search outside the field of conventional drugs and evaluate alternative medicine products for new treatments for diabetic nephropathy.

The extracts of milk thistle, *Silybum marianum*, have been considered as medical remedies since the time of ancient Greece and are now widely used as an alternative medication [5]. It is derived from *Silybum marianum* (milk thistle), an edible plant; it is native to the Mediterranean and grows all through Europe and North America and in India, China, South America, Africa, and Australia [5, 6]. The mechanisms of action of silymarin are not fully understood. Silymarin possesses antioxidant activity. It inhibits lipid peroxidation [7, 8], prevents glutathione depletion [9], and activates antioxidant enzymes that protect DNA from degradation [10]. These properties are determined largely by the presence of β ring catechol group (dihydroxylated β-ring) able to donate hydrogen electrons that stabilize radical species [11]; additionally, the presence of 2,3 unsaturation in conjugation with a 4-oxo-function in the Cring and the presence of functional groups capable of binding transition metal ions, such as iron, may also be responsible for the antioxidant nature of silymarin [12, 13]. In mice, silymarin

administration determined a significant rise in pancreatic and plasma glutathione, prevented lipid peroxidation, and blunts the sustained increment in plasma glucose induced by alloxan [14]. Silymarin administration in streptozotocin treated rats increases the renal activity of several antioxidants enzymes, protecting the kidney from diabetic damage; it decreases podocyte superoxide generation in high glucose-induced models and in vivo in the kidney cortex [14]. Silymarin prevents the damage induced by oxidative agents in AKI [15]. It also prevented glomerular and tubular cell injury and apoptosis in cisplatin- and arsenic-treated rats reducing the ROS generation and apoptosis of tubular cells [16].

An anti-inflammatory effect of silymarin has been described in the liver tissue, in diabetes, or in experimental inflammatory bowel disease; there is evidence that silymarin regulates several inflammatory mediators such as tumoral necrosis factor-alpha (TNF-α), interleukin (IL-1β, IL-6, and IL-1) receptor antagonists, and nitric oxide. Moreover, silymarin downregulates prostaglandin and leukotriene synthesis, two powerful neutrophil chemoattractants, inhibits cyclooxygenase II, additionally reduces the cytotoxic activity and CD8 proliferation, and decreases neutrophil sequestration to the site of inflammation [17].

Oxidative stress and inflammation are considered as major alternative pathways contributing to the pathogenesis of diabetic nephropathy [18]. Silymarin administration in experimental diabetes induced in mice reduced levels of inflammatory cytokines (TNF-α and IL-1β) and oxidative stress mediators like myeloperoxidase activity, lipid peroxidation, carbonyl, and thiol content of pancreatic tissue in an almost dose-dependent manner [17]. In a small randomized controlled trial (RCT) including 60 patients with T2DM and diabetic nephropathy, silymarin reduced urinary and serum TNF-α level compared with placebo; additionally, a significant correlation was found between changes in urinary albumin-creatinine ratio (UACR) and urinary TNF-α level in silymarin-treated patients [19].

Additionally, silymarin possesses antifibrotic properties. It suppresses the expression of profibrogenic procollagen alpha 1 and tissue inhibitor of metalloproteinase-1 (TIMP-1), most likely via downregulation of transforming growth factor-beta 1 (TGF-β1) mRNA in rats with biliary fibrosis [20]. Moreover, it determines a significant reduction of TGF-β [21]. TGF-β plays a key role in the pathogenesis of diabetic nephropathy by mediating glomerulosclerosis and tubulointerstitial fibrosis [22]. It is already demonstrated that its urinary and serum levels are directly correlated with degree of proteinuria and progression of diabetic nephropathy [22]. Silymarin administration determined a reduction in urinary and serum levels of TGF-β in patients with T2DM [23, 24].

This systematic review focuses on the evidence related to silymarin use in diabetes, which is discussed in detail. Therefore, the aim of this meta-analysis was to establish more clearly the benefits of silymarin therapy in patients with diabetes.

2. Why It Is Important to Do the Review

Despite theoretical benefit and efficacy in culture cells of silymarin, a systematic review that included 14 studies found no clear benefits on mortality, improvement in liver histology, or improvements of biochemical markers of liver function in patients with chronic liver disease [25]. To the best of our knowledge there is no systematic review assessing the efficacy of silymarin in diabetes or in renal disease. A number of reviews of complementary and alternative medicine in diabetes were published. A systematic review of Chinese herbs used in T2DM has been published by the Cochrane Library [21], but it includes only one small study involving silymarin [26]. Although the meta-analysis by Suksomboon et al. [27] includes more trials and did not cover silymarin in its scope, the only outcome was glycemic control. This systematic review summarizes the available evidence from RCTs about the effects of silymarin in T2DM.

3. Search Methods for Review

Electronic databases, PubMed, MEDLINE, EMBASE, Cochrane Central Register of Controlled Trials (CENTRAL), AMED (Allied and Complementary Medicine Database), EBM Reviews, ACP Journal Club, and MD Consult, were searched using the terms: milk thistle, *Silybum marianum*, *Silybum*, silymarin, silibinin, silybin, silicristin, silidianin, spelling variants, and diabetes.

4. Types of Participants

4.1. Intervention. Adults (18 years or older) with T2DM were included. Intervention was considered to be included when silymarin based compounds were given. The control group includes placebo or standard care only (any active intervention used with the intention of lowering blood glucose levels, e.g., metformin, sulphonylureas, acarbose, and insulin). Silymarin plus other therapies such as other herbs (*Barberis*) was excluded. Trials were only included if the treatment was given for a minimum of one month. Cointerventions were allowed as long as both arms of the RCT received the same cointervention(s). Only randomized controlled trials were included.

5. Types of Outcome Measures

Consider the following:

(i) Mortality (diabetes-related and all-cause).

(ii) Diabetes complications (neuropathy, retinopathy, nephropathy, chronic kidney disease (CKD) progression, changes in eGFR, and changes in proteinuria).

(iii) Glycemic control (glycated haemoglobin levels (HbA1c) and fasting blood glucose levels).

(iv) Lipid control (changes in cholesterol and triglyceride).

(v) Adverse events.

6. Data Extraction and Management

Data extraction was carried out independently by two authors using standard data extraction forms. Where more than one publication of one study exists, reports were grouped together and the publication with the most complete data

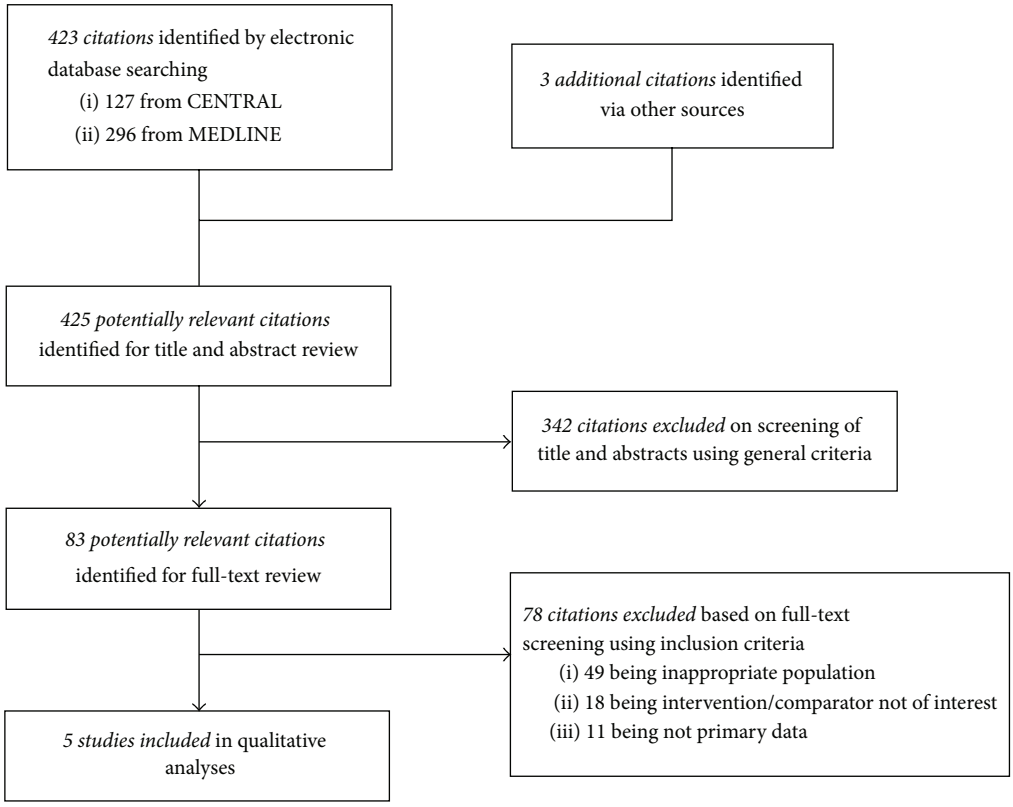

Figure 1: Selection and description of studies.

was used in the analyses. Where relevant outcomes are only published in earlier versions these data were used. Any discrepancy between published versions was highlighted. Risk of bias was assessed using standard domains (Higgins JPT, Green S (editors); Cochrane Handbook for Systematic Reviews of Interventions Version 5.1.0 [updated March 2011]; the Cochrane Collaboration, 2011, available from http://www.cochrane-handbook.org/). We summarized treatment effects using random-effects meta-analysis and expressed results as relative risks (RR) or rate ratios for binary outcomes (mortality, rate of fatal cardiovascular events, and rate of adverse events) or mean difference for continuous outcomes (fasting blood glucose levels, blood pressure, and changes in eGFR) together with 95% CIs. We assessed heterogeneity in treatment estimates using the Cochran Q test and I^2 statistic.

7. Results

7.1. Study Selection. The electronic search identified 423 citations of which we excluded 342 studies based on title and abstract. After reading the full text of the remaining 83 citations we included in our final analysis 5 studies (RCTs) involving 270 patients (Figure 1).

7.2. Study Characteristics. Table 1 shows the key characteristics of the studies and patients included in our systematic review. Four studies evaluated only diabetic patients and one study included patients with diabetes and alcoholic cirrhosis. Follow-up ranged from 45 days to 6 months. Silymarin daily doses ranged between 200 and 600 mg. All studies evaluated short-term outcomes (glycemic control and lipid metabolism). Only one trial reported proteinuria, markers of inflammation and fibrosis while three trials reported malondialdehyde levels, a marker of oxidative stress. CKD progression (change in proteinuria or in creatinine levels or in eGFR were reported by only one study).

7.3. Risk of Bias of the Included Studies

7.3.1. Selection Bias. Two studies [19, 30] were at low risk of selection bias related to random sequence generation and allocation concealment as block randomization procedure (Random Allocation Software (RAS)) was used. Three studies [26, 28, 29] were unclear with respect to selection bias, as the methods used were not clearly described; see Figures 2 and 3.

7.3.2. Detection and Performance Bias. The study by Velussi et al. 1997 [26] was an open control study with high risk of detection and performance bias, while the other included studies were using methods to blind the intervention. Three studies [19, 28, 30] were at low risk for performance bias since blinding was done, and one study [29] was judged at unclear risk for performance bias related to blinding, as the method used was not clearly described and did not report checking of blinding conditions.

Expected results were evaluated by laboratory blood and urine tests; in these circumstances we believe that the risk of

Table 1: Studies included in analysis.

Study	Type of study	Comparison	Study population	Follow-up	Inclusion criteria	Outcomes
Velussi et al. 1997 [26]	12-month open, controlled study	Silymarin plus standard therapy versus standard therapy alone	60 insulin-treated diabetics with alcoholic cirrhosis	4 mo	Age 45 to 70 years (i) NIDDM with alcoholic liver cirrhosis (ii) BMI < 29 kg/m^2 (iii) Ascertained diabetes for a period of at least 5 years and being treated with insulin only (iv) Stable insulin therapy for a period of at least 2 years (v) Negative for markers of hepatitis A, hepatitis B, and hepatitis C and being not addicted to alcohol for a period of at least 2 years prior to the start of the study (vi) No bleeding from varices (vii) Liver biopsy (liver cirrhosis)	Fasting blood glucose Mean daily blood glucose levels Daily glycosuria levels HbA1c Malondialdehyde levels
Huseini et al. 2006 [28]	Randomized double-blind clinical trial	Silymarin plus conventional therapy versus placebo plus conventional therapy	51 patients with type 2 DM	4 mo	Type 2 diabetes according to ADA criteria (2003) (i) Age 40–65 years (ii) Having a fasting blood glucose level less than 250 mg/dL (iii) Duration of diabetes was more than 2 years, and their diabetes was not controlled exclusively by diet	Fasting blood glucose HbA1c Total cholesterol, LDL, and HDL and triglyceride GOT and GPT levels
Hussain 2007 [29]	Randomized, double-blind, placebo-controlled trial	silymarin + glibenclamide versus placebo + glibenclamide versus glibenclamide alone	59 patients with type 2 DM	4 mo	T2DM for at least 5 years (i) Already maintained on 10 mg/day glibenclamide and on diet control (ii) Fasting plasma glucose ≥ 10 mmol/L (iii) HbA1c ≥ 8% (iv) BMI ≥ 29 kg/m^2	Fasting blood glucose HbA1c Body mass index (BMI)
Fallahzadeh et al. 2012 [19]	Randomized, double-blind, placebo-controlled, 2-arm parallel trial	Silymarin versus placebo	60 patients with type 2DM and macroalbuminuria	6 mo	Urinary albumin excretion >300 mg/24 h Treatment of hyperglycemia with (but not limited to) an oral hypoglycemic agent or insulin Treatment of hypercholesterolemia with a statin Presence of diabetic retinopathy	Absolute change in urinary albumin-creatinine ratio Urinary and serum levels of TNF-α Malondialdehyde and TGFβ

Table 1: Continued.

Study	Type of study	Comparison	Study population	Follow-up	Inclusion criteria	Outcomes
Ebrahimpour Koujan et al. 2015 [30]	Randomized, triple-blinded, placebo-controlled clinical trial	Silymarin versus placebo	40 patients with type 2 DM	45 days	Type 2 diabetes patients (i) taking hypoglycaemic medications, (ii) having a body mass index (BMI) between 27 and 35 kg/m^2, and (iii) following a stable habitual diet for the past three months	Antioxidant indices Superoxide dismutase (SOD) and glutathione peroxidase (GPX) activity and total antioxidant capacity C reactive protein

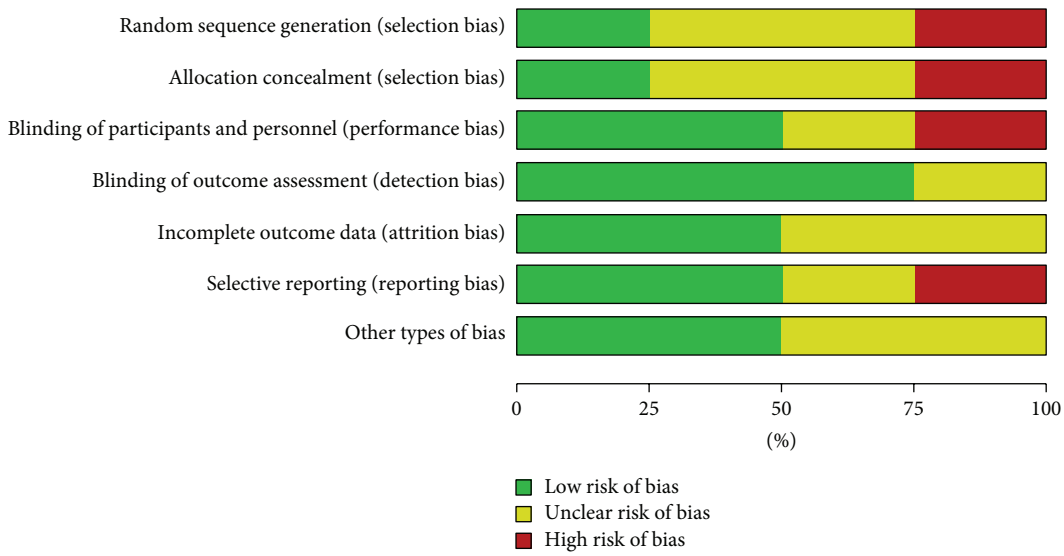

Figure 2: Risk of bias graph: review authors' judgments about each risk of bias item presented as percentages across all included studies.

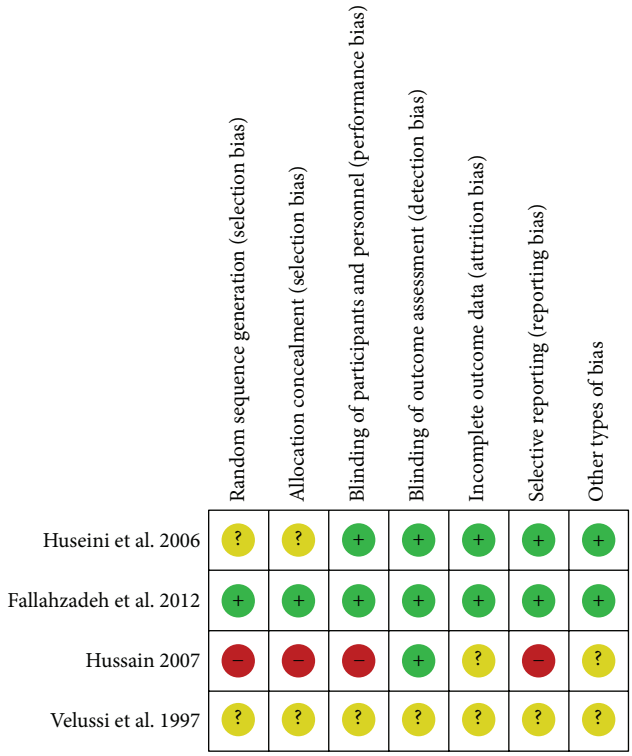

Figure 3: Risk of bias summary: review authors' judgments about each risk of bias item for each included study.

bias is low in four studies [19, 28–30] in terms of detection bias. The study by Velussi et al. 1997 [26] is an open controlled study, and it was considered at a high risk for detection bias. No information about how and if the blinding was done was provided by the authors of the included studies.

7.4. Incomplete Outcome Data. Three studies had a low risk of attrition bias [28–30] as the primary and secondary outcomes were reported for the intention to treat population. By Velussi et al. 1997 [26] it was unclear how many patients were included in the final analysis.

7.5. Selective Reporting Bias. Selective reporting was at low risk in two studies [19, 28] as the main outcomes related to primary disease, stated in the protocol, were reported in the final manuscript. Three studies [26, 29, 30] were with a high risk of reporting bias, based on comparison of reported outcomes in the protocols and the main outcomes related to primary disease and side effects.

7.6. Other Potential Sources of Bias. Two studies [26, 29] were funded by pharmaceutical companies and therefore were judged to be at high risk of bias. For the other three studies we found no other source of bias.

7.7. Outcomes

7.7.1. All-Cause Mortality. Only one study, with 60 patients, reported all-cause mortality [19]; there was only one death in the silymarin group; the cause of death was myocardial infarction probably due to underlying coronary artery disease and was not related to silymarin use.

7.7.2. Diabetes Complications (Neuropathy, Retinopathy, and Nephropathy). We found data only about diabetic nephropathy. One RCT, including 60 patients with T2DM with overt nephropathy, analyzed the efficacy and safety of adding silymarin to RAS inhibitors in reducing progression of diabetic nephropathy [19]. Mean values for changes in serum creatinine were not significantly different between the 2 groups (mean change in silymarin group: 0.021 (−0.027 to 0.07) and in the placebo group: 0.025 (−0.031 to 0.081); difference between groups: −0.004 (−0.076 to 0.069)). Similar results were reported also for eGFR: mean change in the silymarin group: −2.03 (−6.81 to 2.74) mL/min/1.73 m^2 and in the

Table 2: Side effects.

Study	Silymarin group		N	Placebo		N
	Mean	SD		Mean	SD	
Velussi et al. 1997 [26]	2.16 ep/pac/an hypoglycemic event No side effects			2.2 ep/pac/an hypoglycemic event		
Huseini et al. 2006 [28]	Not reported					
Hussain 2007 [29]	No side effects					
Fallahzadeh et al. 2012 [19]	Nausea and vomiting	3 (10%)	28	Nausea and vomiting	2 (6.7%)	28
	Headache	2 (6.7%)	28	Headache	0 (0%)	28
	Dyspepsia and bloating	1 (3.3%)	28	Dyspepsia and bloating	0 (0)	28
Ebrahimpour Koujan et al., 2015 [30]	No side effects					

placebo group: −1.81 (−5.75 to 2.14) mL/min/1.73 m^2; difference between groups: −0.23 (−6.28 to 5.82) mL/min/1.73 m^2.

Changes in proteinuria were also analyzed in this trial. Mean UACR levels decreased in both groups: −566 (−827 to −305) mg/g in the silymarin group versus −219 (−454 to 16) mg/g for placebo. However, this decrement was significantly higher in the silymarin group. Moreover, at the end of the treatment phase, UACR decreased more than 50% from baseline in 12 patients from the silymarin group compared with 6 patients from the placebo group ($P = 0.09$).

7.7.3. Glycemic Control. Silymarin administration was associated with a significant reduction in fasting blood glucose levels (mean difference [MD] (−26.86 mg/dL; 95% CI [−35.42, −18.30])) in four trials [19, 26, 28, 29]. Similarly, compared with placebo, silymarin administration reduced significantly HbA1c levels ([MD] 1.07; 95% CI [−1.73–0.40]); see Figure 4.

7.7.4. Lipid Control. Three studies reported data on this outcome [19, 26, 29]. No difference was found between the two arms: MD for cholesterol levels was −2.48 mg/dL; 95% CI −23.14–18.18; MD for HDL cholesterol was −5.27 mg/dL; 95% CI −24.20–13.66; MD for triglyceride was 13.87 mg/dL; 95% CI −9.12–36.67; see Figure 5.

7.7.5. Adverse Events. Adverse events were reported only in two studies; except for the gastrointestinal disturbances and headache (data reported in one study), silymarin was found to be safe and without major side effects (see Table 2).

8. Discussion

In low- to very low-quality evidence from 5 RCTs trials done on 270 patients, routine silymarin administration in patients with T2DM might improve the glycemic control, has no effect on lipid profile, and has imprecise effects on CKD. Adverse effects were not reported systematically.

In the last ten years, silymarin was gradually recognized as hopeful complementary medication in diabetes. Silymarin treatment resulted in a statistically significant improvement in glycemic control in four studies compared with placebo. Heterogeneity was also observed in the study results. This may be due to differences in the dose of milk thistle used and in the treatment regimens. Besides, huge difference in baseline fasting blood glucose level may also play a part. However, although hyperglycemia was associated with an increased mortality and CV risk in epidemiological and pathophysiological studies in patients with T2DM [31], the association between the extent of glucose lowering and the reduction in CV risk is less well defined. Clinical trials evaluating the effect of intensive glycemic control on main outcomes in type 2 DM patients showed disappointing results. Results of the main Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial indicate that a therapeutic strategy targeting HbA1c levels below 6.0% increased the rate of death from any cause (as compared with the standard-therapy group, the intensive-therapy group had a relative increase in mortality of 22% and an absolute increase of 1.0% during this follow-up period, with similar differences in death from CV causes) [32]. Moreover, results from a recently published secondary analysis of the ACCORD, including patients with mild-to-moderate CKD, were disappointing [33]. An intensive glycemic control was associated with a 41% increase in CV mortality and a 31% increase in all-cause mortality [33]. A recent systematic review with both meta-analysis and trial-sequential analysis of randomized clinical trials conducted by Hemmingsen et al. showed no meaningful reduction in major outcomes with intensive glycemic control in patients with type 2 DM [34]. In this meta-analysis (including 28,614 participants with type 2 DM from 20 RCTs), intensive glycemic control did not reduce all-cause mortality (RR 1.02; 95% CI 0.91–1.13). Additionally, it did not reduce the risk of CV mortality (RR 1.11; 95% CI 0.92–1.35). Intensive treatment reduced the risk for nonfatal MI (RR 0.85, 95% CI 0.76–0.95, and $P = 0.004$) in meta-analysis, but this was not confirmed in trial-sequential analysis. Furthermore, reduction in nephropathy was not significant (RR 0.83; 95% CI 0.64–1.06). Moreover, intensive control of blood glucose increases patients' relative risk of severe hypoglycemia by 30%. Patients with type 2 DM have a diversity of lipid abnormalities including high levels of chylomicron remnants, enlarged levels of LDL, and low levels of HDL [35]. Dyslipidaemia is a main risk factor for macrovascular complications in diabetes patients. Multiple clinical trials have showed favourable effects of lipid control on CVD outcomes in diabetic subjects with CHD and for primary CV prevention [36, 37]. A recent meta-analysis, including data

Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Mean difference IV, random, 95% CI
1.1.1 HbA1c								
Huseini et al. 2006	6.78	1.05	25	9.45	2.16	25	18.7%	−2.67 [−3.61, −1.73]
Fallahzadeh et al. 2012	−0.18	0.72	28	−0.19	1.2379	28	25.2%	0.01 [−0.52, 0.54]
Hussain 2007	7.45	0.8	18	8.71	0.6261	20	26.3%	−1.26 [−1.72, −0.80]
Velussi et al. 1997	7.2	0.2	30	8	0.3	30	29.8%	−0.80 [−0.93, −0.67]
Subtotal (95% CI)			**101**			**103**	**100.0%**	**−1.07 [−1.73, −0.40]**
Heterogeneity: $\tau^2 = 0.38$; $\chi^2 = 27.84$; df = 3 ($P < 0.00001$); $I^2 = 89\%$								
Test for overall effect: $Z = 3.15$ ($P = 0.002$)								
1.1.2 Fasting Glucose								
Huseini et al. 2006	133	39	25	188	48	26	10.0%	−55.00 [−78.96, −31.04]
Fallahzadeh et al. 2012	−3.43	49.9536	28	−11	57.252	28	7.7%	7.57 [−20.57, 35.71]
Hussain 2007	167.58	2.34	18	193.14	3.6	20	45.0%	−25.56 [−27.47, −23.65]
Velussi et al. 1997	165	1	30	193	17	30	37.3%	−28.00 [−34.09, −21.91]
Subtotal (95% CI)			**101**			**104**	**100.0%**	**−26.86 [−35.42, −18.30]**
Heterogeneity: $\tau^2 = 41.39$; $\chi^2 = 11.67$; df = 3 ($P = 0.009$); $I^2 = 74\%$								
Test for overall effect: $Z = 6.15$ ($P < 0.00001$)								
Test for subgroup differences: $\chi^2 = 34.71$; df = 1 ($P < 0.00001$); $I^2 = 97.1\%$								

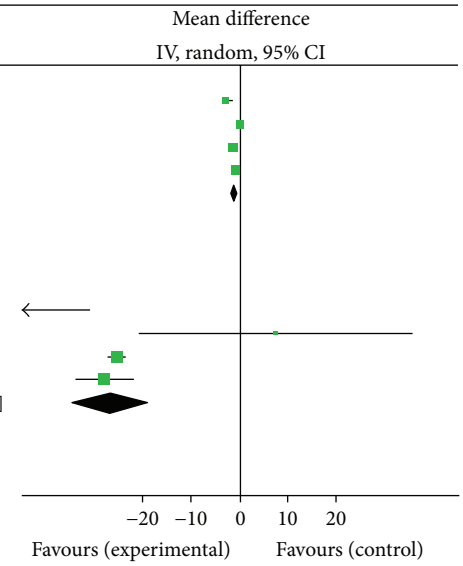

Figure 4: Glycemic control.

Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Mean difference IV, random, 95% CI
1.2.1 Total Cholesterol								
Huseini et al. 2006	198	41	25	215	50	26	26.8%	−17.00 [−42.05, 8.05]
Fallahzadeh et al. 2012	−22.9	48.9994	28	−12.2	33.268	28	29.5%	−10.70 [−32.64, 11.24]
Velussi et al. 1997	162	9	30	150	5	30	43.7%	12.00 [8.32, 15.68]
Subtotal (95% CI)			**83**			**84**	**100.0%**	**−2.48 [−23.14, 18.18]**
Heterogeneity: $\tau^2 = 251.05$; $\chi^2 = 8.83$; df = 2 ($P = 0.01$); $I^2 = 77\%$								
Test for overall effect: $Z = 0.24$ ($P = 0.81$)								
1.2.2 Triglyceride								
Huseini et al. 2006	211	136	25	207	93	26	10.7%	4.00 [−60.19, 68.19]
Fallahzadeh et al. 2012	−10.3	60.3467	28	−6.04	47.8647	28	32.6%	−4.26 [−32.79, 24.27]
Velussi et al. 1997	154	26	30	128	19	30	56.7%	26.00 [14.48, 37.52]
Subtotal (95% CI)			**83**			**84**	**100.0%**	**13.78 [−9.12, 36.67]**
Heterogeneity: $\tau^2 = 206.10$; $\chi^2 = 4.00$; df = 2 ($P = 0.14$); $I^2 = 50\%$								
Test for overall effect: $Z = 1.18$ ($P = 0.24$)								
1.2.3 HDL Cholesterol								
Huseini et al. 2006	61	19	25	85	91	26	21.0%	−24.00 [−59.76, 11.76]
Fallahzadeh et al. 2012	0.32	10.5736	28	0.61	8.9488	28	79.0%	−0.29 [−5.42, 4.84]
Velussi et al. 1997	0	0	0	0	0	0		Not estimable
Subtotal (95% CI)			**53**			**54**	**100.0%**	**−5.27 [−24.20, 13.66]**
Heterogeneity: $\tau^2 = 111.19$; $\chi^2 = 1.65$; df = 1 ($P = 0.20$); $I^2 = 40\%$								
Test for overall effect: $Z = 0.55$ ($P = 0.59$)								
Test for subgroup differences: $\chi^2 = 1.73$; df = 2 ($P = 0.42$); $I^2 = 0\%$								

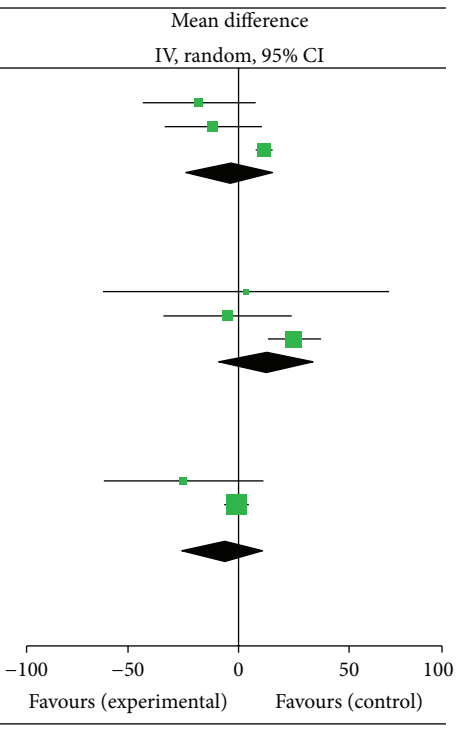

Figure 5: Lipid control.

from over 18,000 patients with diabetes from 14 randomized trials (mean follow-up 4.3 years), showed a 9% proportional reduction in all-cause mortality and 13% reduction in vascular mortality, for each mmol/L reduction in LDL cholesterol [37]. Treatment with silymarin did not successfully improve the lipid profile markers in our systematic review. Disparate data are provided by several experimental or human studies. Silymarin administration in rats with impaired lipid profile determines a significant reduction in LDL, VLDL, triglyceride, and cholesterol with elevation of HDL cholesterol [38]. Silymarin seems to decrease the intracellular cholesterol esterification (by diminishing acyl CoA enzyme activity). Silymarin inhibits HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase enzyme and reduces the cholesterol synthesis [39]. Moreover, silymarin partially antagonizes the increase in liver content of triglycerides, decrease in VLDL

synthesis, and the availability of free VLDL secretion in the intestine [40, 41]. Additionally, it reduces lipid accumulation by downregulating adipogenic factors, such as peroxisome proliferator-activated receptor γ (PPARγ), CCAAT-enhancer binding protein α (C/EBPα), and fatty acid-binding protein 4 (FABP4) [42]. However, human studies showed different results. Several small human studies [40, 41] confirmed the benefit reported by experimental studies, while other small studies did not confirm a significant improvement in lipid metabolism after silymarin use.

Further studies are necessary before a firm conclusion can be made. No unquestionable data regarding the effects of silymarin on main outcome (mortality or progression of diabetic kidney disease) are available. Only one small study with a short duration of the treatment phase showed a reduction of proteinuria in patients with type 2 diabetes with overt nephropathy. Inhibition of inflammatory mediators and attenuation of oxidative stress may be the possible mechanisms behind this observed efficacy.

This systematic review has a number of potential limitations. First, the small number of studies with a small sample size and short-term follow-up limits the power of our meta-analyses. The different silymarin products (without specific details of formulations used) and different dosage regimens, treatment durations, and endpoints used also make drawing meaningful comparisons between studies difficult. Furthermore, it is not known how surrogate outcomes, such as glycemic control, can be translated into patient-relevant outcomes including progression to end-stage renal disease and mortality. This warrants further investigation.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Upregulation of $\alpha 3\beta 1$-Integrin in Podocytes in Early-Stage Diabetic Nephropathy

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Background. Podocyte injury plays an important role in the onset and progression of diabetic nephropathy (DN). Downregulation of $\alpha 3\beta 1$-integrin expression in podocytes is thought to be associated with podocyte detachment from the glomerular basement membrane, although the mechanisms remain obscure. To determine the mechanism of podocyte detachment, we analyzed the expression levels of $\alpha 3\beta 1$-integrin in podocytes in early and advanced stages of DN. *Methods*. Surgical specimens from DN patients were examined by *in situ* hybridization, and the expression levels of $\alpha 3$- and $\beta 1$-integrin subunits in glomeruli of early ($n = 6$) and advanced ($n = 8$) stages were compared with those of normal glomeruli ($n = 5$). Heat-sensitive mouse podocytes (HSMP) were cultured with TGF-$\beta 1$ to reproduce the microenvironment of glomeruli of DN, and the expression levels of integrin subunits and the properties of migration and attachment were examined. *Results*. Podocytes of early-stage DN showed upregulation of $\alpha 3$- and $\beta 1$-integrin expression while those of advanced stage showed downregulation. Real-time PCR indicated a tendency for upregulation of $\alpha 3$- and $\beta 1$-integrin in HSMP cultured with TGF-$\beta 1$. TGF-$\beta 1$-stimulated HSMP also showed enhanced *in vitro* migration and attachment on collagen substrate. *Conclusions*. The results suggested that podocyte detachment during early stage of DN is mediated through upregulation of $\alpha 3\beta 1$-integrin.

1. Introduction

Diabetic nephropathy (DN) is one of the major indications for hemodialysis treatment in patients with diabetes mellitus (DM). It is important to elucidate the cause of DN and develop more effective treatments based on the escalating medical costs and need for improvement of quality of life (QOL) of DN patients. Previous studies showed that the onset and progression of DN correlate with injury of glomerular epithelial cells (podocytes) [1–7]. Furthermore, effacement of foot process followed by podocyte detachment from the glomerular basement membrane (GBM) is known to result in proteinuria [8]. Among the many molecules that are known to be involved in cell-to-cell and cell-to-substrate attachment in glomeruli, $\alpha 3\beta 1$-integrin is expressed primarily in podocytes and considered to play a critical role in the attachment of podocytes to the GBM [9, 10]. Several studies have so far examined changes in $\alpha 3\beta 1$-integrin expression in podocytes in patients with various types of nephropathies and equivalent animal model [11–19]. The results of studies involving DN patients, animal models of DN, and *in vitro* culture of podocytes under high glucose conditions have so far demonstrated underexpression of $\alpha 3$-integrin subunit in podocytes and suggested that the main cause of podocyte detachment was a decrease in the number of $\alpha 3\beta 1$-integrins on the surface of podocytes [20–23]. However, the mechanism responsible for the downregulation of $\alpha 3\beta 1$-integrin during progression of DN remains obscure.

TGF-$\beta 1$ is synthesized and secreted by glomerular mesangial cells (MC) in patients with nephropathy and animal models of glomerulonephritis [24]. The secreted TGF-$\beta 1$ from MC stimulates podocyte to synthesize collagen type IV

Table 1: Baseline characteristics.

	NHK ($n = 5$)	DN1 ($n = 6$)	DN2 ($n = 8$)
Gender (M/F)	4/1	4/2	6/2
Age (years)	45 ± 10	44 ± 17	48 ± 12
Serum creatinine (mg/dL)	0.9 ± 0.2	0.7 ± 0.1	1.1 ± 0.3*
Total protein (g/dL)	7.4 ± 0.8	6.9 ± 0.6	6.0 ± 1.6
HbA1c (%)	ND	8.7 ± 2.3	8.8 ± 2.9
Urinary protein (g/day)	ND	0.22 ± 0.15	1.95 ± 2.45*
Creatinine clearance (mL/min)	ND	97.0 ± 20.5	73.3 ± 26.0*

Data are expressed as mean ± SD.
* $p < 0.05$ versus DN1.
ND: not determined.

[25, 26]. Furthermore, TGF-β1 is considered to be one of the most important factors involved in the reproduction of microenvironment of DN glomeruli in culture systems [27, 28], though almost all *in vitro* studies have been performed using podocytes cultured under high glucose conditions.

The present study was designed to determine the mechanism of podocyte detachment in DN. For this purpose, we examined the expression of α3- and β1-integrin subunits in glomeruli of patients with early and advanced stages of DN. We also reproduced the changes, including changes in the expression levels of $\alpha 3\beta 1$-integrin in an *in vitro* culture of heat-sensitive mouse podocytes (HSMP). Contrary to the data of previous studies [20–23], the results showed upregulation of $\alpha 3\beta 1$-integrin expression in podocytes in early-stage DN. The results were confirmed in *in vitro* culture of TGF-β1-stimulated mouse podocytes.

2. Methods

2.1. Subjects. Renal biopsy tissues of 14 patients with DN were used. The diagnosis of DN was confirmed by histopathological examination and the severity was classified into two grades: grade I (DN1 = 6 patients) reflected mild mesangial expansion and DN grade II (DN2 = 8 patients) represented moderate mesangial expansion. Control samples were obtained from uninvolved portions of surgically removed kidneys afflicted with malignancies (5 patients). After resection, the samples were embedded in optimal cutting temperature (OCT) compound (Tissue Tek; Miles, Elkhart, IN) and stored at −70°C until use. Blood and urine samples were collected from subjects immediately before renal biopsy. Serum creatinine, total protein, HbA1c, urinary protein, and creatinine clearance were measured using standard methods in our hospital (Table 1).

This study was reviewed and approved by the Institutional Review Board of Tokai University School of Medicine. All procedures were performed in accordance with the principles of the Declaration of Helsinki. Informed consents were obtained from all subjects.

2.2. In Situ Hybridization. Oligonucleotide probes for mRNAs of human α3- and β1-integrin subunits were labeled using a digoxigenin (DIG) oligonucleotide tailing kit according to the standard protocol (Boehringer Mannheim, Mannheim, Germany). Free DIG was removed by ethanol precipitation and probes were dissolved in diethylpyrocarbonate-treated water.

In situ hybridization was performed according to a protocol developed in our laboratory with minor modifications. Briefly, sections of specimens were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and then deproteinized with HCl and digested with proteinase K (Sigma Chemical, St. Louis, MO). Following a treatment with a hybridization buffer, specimens were hybridized overnight with a DIG-labeled oligonucleotide probe in the same buffer. After washing with a stringent condition, the DIG-labeled probe was detected immunohistochemically using a mouse monoclonal anti-DIG antibody (Boehringer Mannheim), horseradish peroxidase- (HRP-) conjugated rabbit anti-mouse IgG antibody (Dako, Glostrup, Denmark), and HRP-conjugated swine anti-rabbit IgG antibody (Dako). The reaction was visualized with diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl, pH 7.6, and 0.03% H_2O_2. Sections were briefly counterstained with hematoxylin, rinsed, dehydrated, cleared in xylene, and mounted. Three independent investigators who were blinded to the results of histopathological classification counted the numbers of stained cells. The percentage of mRNA-positive cells relative to the total glomerular cells was determined.

2.3. Cell Culture. HSMP were cultured with RPMI 1640 medium (Nissui Pharma, Tokyo, Japan) containing 10% fetal bovine serum, 50 U/mL penicillin, and 50 mg/mL streptomycin in 5% CO_2-95% air atmosphere. Cells proliferated at 33°C in the presence of 50 units/mL mouse recombinant IFN-γ (Aviva System Biology, San Diego, CA). For podocyte differentiation, cells between passages 10 and 13 were cultured at 37°C without IFN-γ for two weeks. Podocyte differentiation was confirmed by the expression of synaptopodin detected by quantitative real-time PCR. To set the conditions of microenvironment to mimic those of DN glomeruli, 1 ng/mL TGF-β1 (R&D Systems, Minneapolis, MN) was added to medium. The medium was replaced with a new one every other day.

2.4. Attachment and Migration Assays. HSMP were differentiated as above, and 1 ng/mL TGF-β1 was added before three days of experiments. Cells were harvested by trypsinization, counted, and inoculated into plates as described below.

To measure the ability of attachment of differentiated HSMP to the substrate, 1×10^4 cells per well were inoculated into 24-well plate coated with collagen type IV and incubated at 37°C. After 30-minute incubation, the plates were shaken to detach weakly binding cells, washed several times with PBS(−), refilled with culture medium, and incubated at 37°C in 5% CO_2. The next day the cells were fixed with 4% paraformaldehyde and photographed. The number of cells in the view fields was counted.

To measure the ability of differentiated HSMP to migrate towards the substrate, the Oris Pro cell migration assay kit (Platypus Technology, Fitchburg, WI) was used. 4×10^4

cells per well were inoculated into 96-well plate coated with collagen type I supplied with the kit. After 24 hours, the cells were fixed with 4% paraformaldehyde and stained with hematoxylin. Absorbance of 600 nm light passed into the central detection zone was measured by spectrophotometry (Infinite F200PRO, TECAN, Männedorf, Switzerland).

2.5. Quantitative Real-Time Polymerase Chain Reaction. Differentiated HSMP were inoculated into wells of 48-well plate coated with collagen type IV with a rate of 1×10^6 per well and cultured with 1 ng/mL TGF-β1 at 37°C under 5% CO_2-95% air atmosphere. According to the time schedule, total RNAs were recovered from cells using the RNAqueous RNA purification kit (ThermoFisher, Waltham, MA) and converted to cDNA using SuperScript III First-Strand Synthesis System (ThermoFisher). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the cDNA, as described previously [29]. The reaction mixture was prepared as described in the instruction manual of the kit (TaqMan Gene Expression Assays; ThermoFisher) that also contained the primers and probes for mouse α3-integrin (assay ID: Mm00442910_m1), β1-integrin (assay ID: Mm01253230_m1), and 18S ribosomal RNA (rRNA), which was used as the endogenous control. PCR was performed on an ABI PRISM 7500 (ThermoFisher). Data were analyzed by the comparative Ct method, and the amounts of integrin mRNAs were expressed relative to that of 18S rRNA.

2.6. Statistical Analysis. Results are expressed as mean ± SD. Differences between two groups were assessed by Student's t-test. Spearman's correlation coefficient analysis was used to examine the relationship between parameters. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Patient Characteristics. The clinical characteristics of the patients are shown in Table 1. Based on the degree of mesangial expansion, patients were categorized into the DN1 and DN2 groups, as described in Methods. Serum creatinine, urinary protein, and creatinine clearance worsened significantly during the course of DN in DN2 patients compared with DN1 patients.

3.2. Expression of Integrin Subunits Detected by In Situ Hybridization. Representative glomeruli stained for α3- and β1-integrin are shown in Figure 1 and the percentages of cells positive for these mRNAs are summarized in Table 2. Although the percentage of cells positive for each integrin subunit in glomeruli was not significantly different to those of NHK when data of all DN patients were analyzed, those of DN1 were significantly higher (Table 2). Particularly, the percentage of cells positive for β1-integrin mRNA was significantly higher in DN1 than NHK. Furthermore, the percentages of cells positive for α3- and β1-integrin subunits were both significantly lower in DN2. Further analysis showed strong correlation between percentages of cells positive for α3- and β1-integrin subunits (Figure 2), suggesting that the increase in the subunits resulted in the formation of $\alpha 3\beta 1$-integrin on the podocyte surface.

Table 2: Percentages of cells positive for integrin mRNAs in glomeruli.

	NHK ($n = 5$)	Patients with diabetic nephropathy		
		Total ($n = 14$)	DN1 ($n = 6$)	DN2 ($n = 8$)
α3-integrin (%)	14.1 ± 2.8	14.9 ± 4.9	17.9 ± 4.4	12.0 ± 3.5*
β1-integrin (%)	12.6 ± 4.4	15.5 ± 3.5	17.6 ± 2.6	13.3 ± 3.0¶

Data are expressed as mean ± SD.
*$p < 0.05$ versus DN1.
¶$p < 0.01$ versus DN1.

3.3. Induction of α3- and β1-Integrin Expression in Cultured Mouse Podocytes. Next, we induced the expression of integrin subunits in DN1 glomeruli in cultured HSMP. To mimic the condition of DN glomerular microenvironment, the cultured cells were treated with 1 ng/mL TGF-β1 for three days and RNAs were recovered at various time points. Although the expression of α3-integrin gene decreased significantly at the onset of the experiment, it recovered after 48 hours of culture and finally reached about twice the expression level at baseline (0 hours) (Figure 3). On the other hand, the expression of β1-integrin gene increased steadily and reached more than three times the baseline expression level at 72 hrs (Figure 3). Thus, the expression levels of both integrins increased proportionally with culture duration in the presence of TGF-β1. These results demonstrated that induction of α3- and β1-integrin subunits in glomeruli of DN1 patients can be reproduced by HSMP cultured with TGF-β1. On the other hand, enhanced detachment of cells from substrate was not observed during the experiments, and TGF-β1 did not affect the number of cells under these conditions (data not shown).

3.4. TGF-β1 Enhances Substrate Attachment and Migration of HSMP. Finally, we examined the effects of TGF-β1-induced expression of $\alpha 3\beta 1$-integrin on substrate attachment and cell migration. For substrate attachment, type IV collagen-coated plates were shaken and washed after 30 minutes of cell inoculation, and the number of remaining cells was counted in the presence or absence of TGF-β1. On the other hand, migration was measured by using a cell migration assay kit, in which the extent of migration was measured by absorbance of stained cells that entered the central clear space of the plates. TGF-β1 significantly enhanced HSMP attachment to type IV collagen (Figure 4) and their migration on type I collagen-coated plate (Figure 5). These results suggest the involvement of both processes in the increased expression of $\alpha 3\beta 1$-integrin induced by TGF-β1.

4. Discussion

The main finding of the present study was upregulation of integrin expression in podocytes of patients with early DN and in HSMP cultured with TGF-β1.

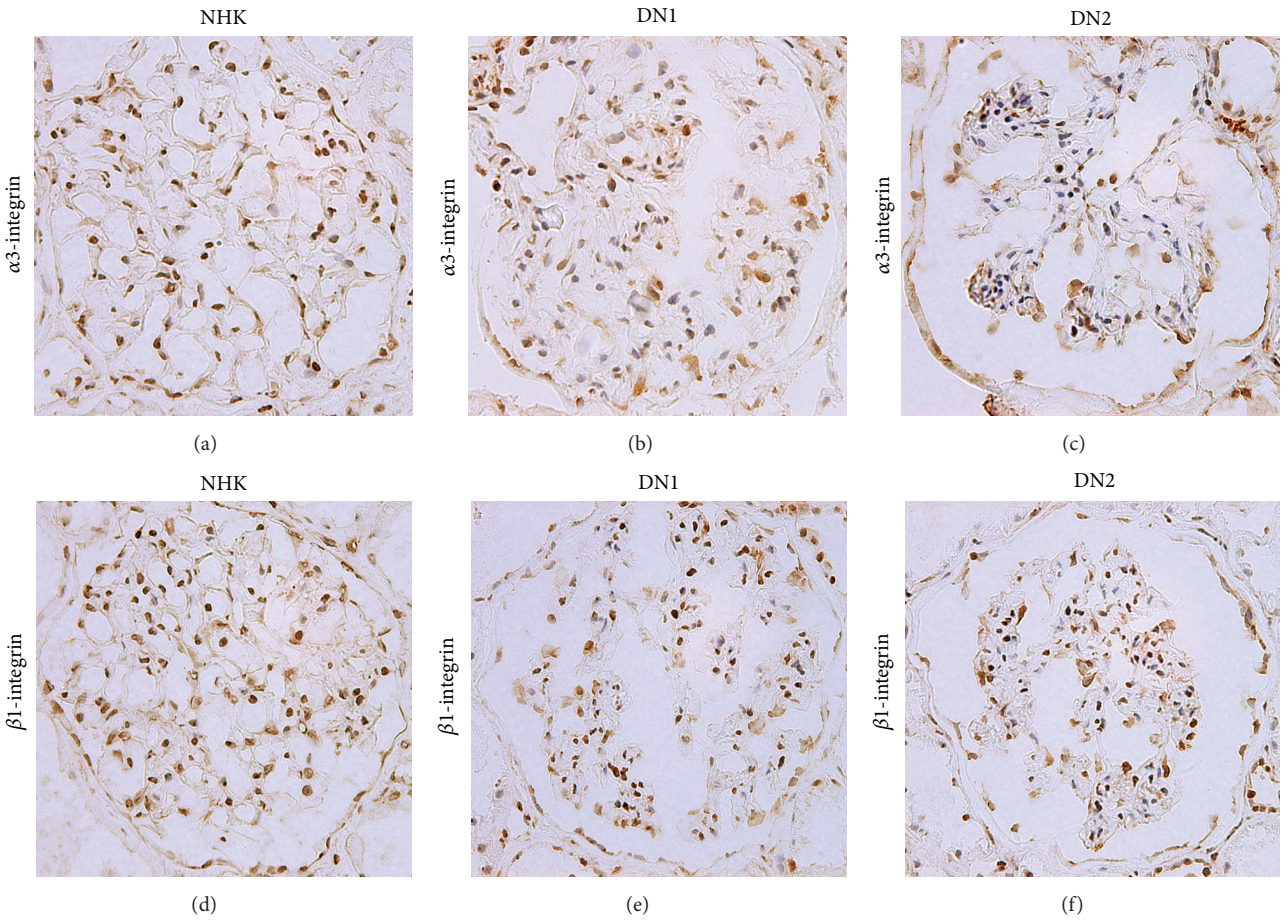

Figure 1: *In situ* hybridization of α3- and β1-integrin subunits. Representative mRNA expression of integrin subunits in glomeruli of normal human kidney, early (DN1) and advanced (DN2) stages of DN. Integrin expression is colored by DAB (brown), and nuclei of cells are stained by hematoxylin (blue) (magnification, ×100).

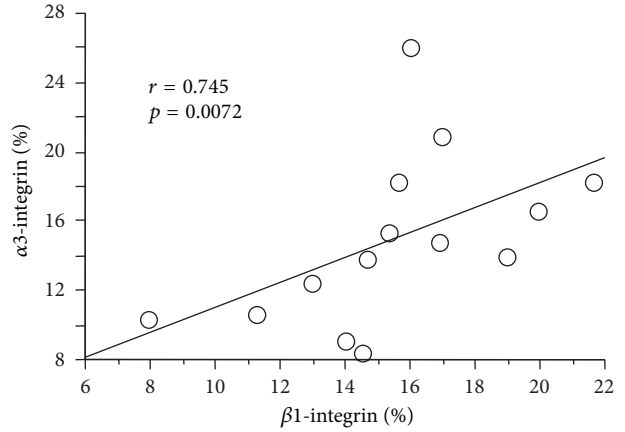

Figure 2: Strong correlation between the expression levels of α3- and β1-integrin subunits in DN glomeruli of 14 DN patients. The percentages of cells positive for α3- and β1-integrin in glomeruli were examined by *in situ* hybridization.

Since no proliferation of mature podocytes is noted in the majority of nephropathies, including DN [30], integrin mRNA overexpression in podocytes, including higher percentage of integrin-positive cells in DN1 glomeruli, can be detected by *in situ* hybridization. Although several studies involving DN patients and animal models reported a decrease or no change in $\alpha3\beta1$-integrin expression in podocytes, almost all of them assessed the expression of integrin in podocytes of proceeded stage of DN in patients and animals [20–23]. The present study also showed no significant change in α3- and β1-integrin expression when data of all DN patients were pooled together and compared with those of NHK (Table 2). On the other hand, separate analysis of data of patients with early stage (DN1) demonstrated significant upregulation of integrin, which could have been masked in previous studies. Interestingly, controversial results regarding the expression patterns of integrin in podocytes have been reported in various types of nephropathies and glomerulonephritis other than DN [11–19]. In general, proteinuria correlates with downregulation of integrin in podocytes in various nephropathies, although an increase or no change in integrin expression has been reported in some cases of early-stage nephropathy. For example, overexpression of glomerular α3-integrin was reported in a rat model of minimal-change nephropathy 10 days after injection of puromycin

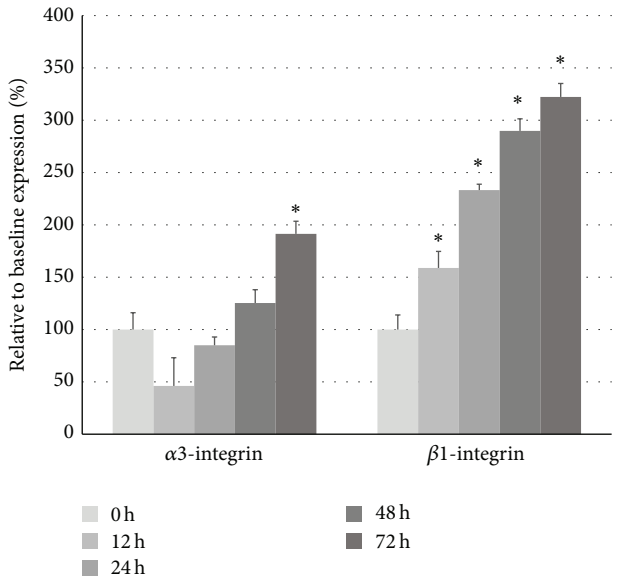

Figure 3: Expression levels of α3- and β1-integrin subunits in cultured mouse podocytes (HSMP) treated with TGF-β1. Differentiated HSMP were cultured with 1 ng/mL TGF-β1, and the expression levels of α3- and β1-integrin were measured by semiquantitative real-time PCR. The relative mRNA level at each time point is represented as a percentage of the baseline value (0 hours). $^*P < 0.05$ versus the baseline value.

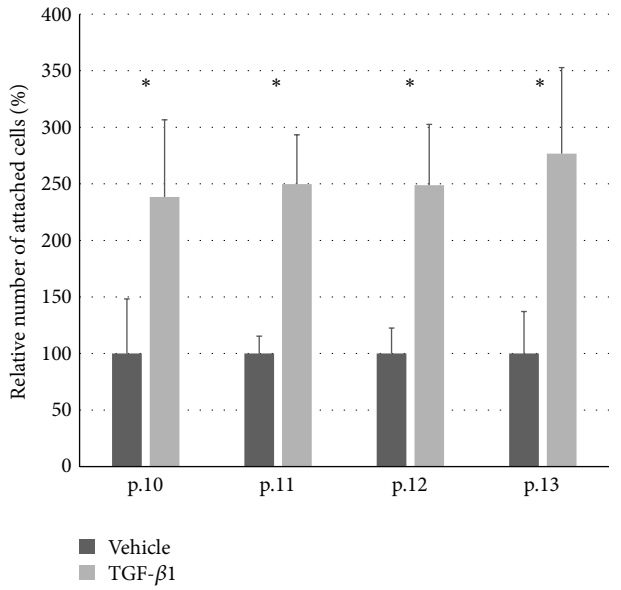

Figure 4: Attachment assay of HSMP cultured with/without TGF-β1. Attachment assay was performed as described in Methods using cells of passages 10–13. The y-axis shows percentage of attached HSMP cultured with TGF-β1 relative to that of HSMP cultured with the vehicle. $^*P < 0.05$ versus vehicle.

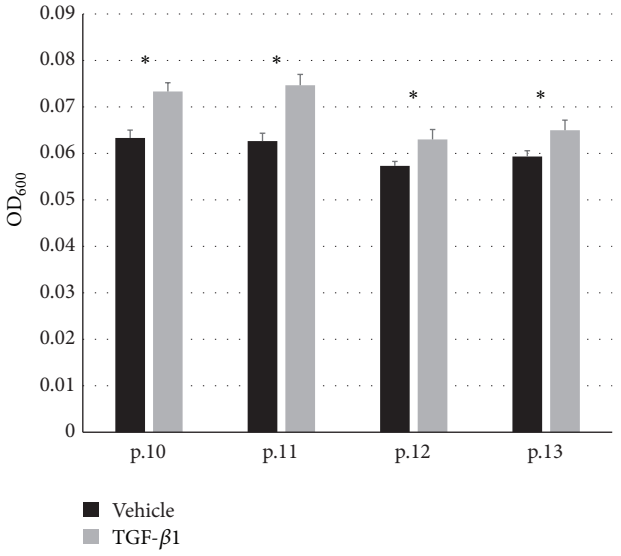

Figure 5: Migration assay of HSMP cultured with/without TGF-β1. Migration assay was performed as described in Methods using cells of passages 10–13. Absorbance at 600 nm (OD_{600}) of migrated HSMP stained with hematoxylin. $^*P < 0.05$ versus vehicle.

aminonucleoside (PAN) [17]. Furthermore, Baraldi et al. reported downregulation of α3-integrin in 6 patients with stage I-III membranous nephropathy [11], whereas Bains et al. reported little change in integrin expression in 18 patients with "early-" stage proteinuria [16] and Chen et al. used a rat model of streptozotocin-induced DN to report no change in α3-integrin expression within the first week followed by a decrease after 1–3 months [21].

The upregulated expression of integrin in DN1 podocytes probably represents increased turnover and reconstruction of adhesion structures responsible for cell-substrate interaction [16]. This argument is supported by the finding of enhanced attachment and migration of HSMP with upregulated integrin expression. The reconstruction of adhesion structures could be a mechanism to counteract increased instability of DN1 podocytes caused by the effacement of foot process.

In the present study, HSMP were cultured with TGF-β1 instead of high glucose concentrations to reproduce the microenvironment in DN glomeruli. Previous studies reported that high glucose concentrations in culture medium result in downregulation of α3-integrin expression in podocytes within 24 hours, although the induction of α3-integrin as observed in DN1 podocytes has not been reported [21, 22]. It is likely that hyperglycemia does not directly change integrin expression in the early stages of DN. On the other hand, TGF-β1 is produced abundantly in mesangial proliferative glomerulonephritis [24, 27, 28] and induces podocytes to synthesize collagen type IV, which contributes to the proliferation of GBM [25, 26]. Moreover, TGF-β1 induces MC to synthesize type I collagen and β1-integrin, which contribute to the proliferation of mesangial substrates [31]. These evidences suggest that TGF-β1, rather than hyperglycemia, is directly involved in the regulation of cell-substrate interaction of podocytes and MC in glomeruli.

TGF-β1 is also known to induce cell death in certain types of cells [32, 33]. Our results showed death of HSMP after their detachment from the substrate when they were

cultured with TGF-β1 at concentrations higher than that used in experiments of the present study (data not shown). Although upregulation of genes involved in cell death of podocytes cultured under high glucose concentrations was reported [34], some studies described maintaining cultured podocytes under high glucose concentrations as long as 1-2 weeks and even more than 6 months [35, 36]. These studies suggest that direct stimulation by high glucose concentration may not be sufficient to induce podocyte detachment. One can probably establish a culture system that allows the process of detachment and death of podocytes.

In conclusion, upregulation of $\alpha 3\beta 1$-integrin expression was detected in early-stage DN, suggesting reconstruction of adhesion structures essential for cell-substrate interaction after effacement of foot process. HSMP cultured with TGF-β1 reproduced α3- and β1-integrin upregulation observed in DN1, and podocytes stimulated with TGF-β1 showed enhanced attachment and migration. Our *in vitro* culture system that mimics the onset and progression of DN can be used to elucidate the mechanism of podocyte detachment and to develop effective treatments for proteinuria.

Competing Interests

The authors declare no conflict of interests in relation to this paper.

Acknowledgments

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Research Article

Implementation of a Diabetes Educator Care Model to Reduce Paediatric Admission for Diabetic Ketoacidosis

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Introduction. Diabetic Ketoacidosis (DKA) is a serious complication that can be life-threatening. Management of DKA needs admission in a specialized center and imposes major constraints on hospital resources. *Aim*. We plan to study the impact of adapting a diabetes-educator care model on reducing the frequency of hospital admission of children and adolescents presenting with DKA. *Method*. We have proposed a model of care led by diabetes educators for children and adolescents with diabetes. The team consisted of highly trained nurses. The model effectiveness is measured by comparing the rate of hospital admission for DKA over 4-year period to the baseline year prior to implementing the model. *Results*. There were 158 admissions for DKA over a 5-year period. Number of patients followed up in the outpatient diabetes clinics increased from 37 to 331 patients at the start and the end of the study years. Admission rate showed a downward trend over the five-year period. Percentage of admission for DKA is reduced from 210% to 1.8% (P 0.001). *Conclusion*. Diabetes educator care model is an effective and a sustainable measure to reduce hospital admission for DKA in children and adolescents.

1. Introduction

Diabetic Ketoacidosis (DKA) is a common but a preventable complication. It is associated with a significant potential for long-term morbidity and mortality. It is estimated that the risk of DKA in established type 1 diabetes patients can be up to 10% per patient per year [1]. In national population studies, the mortality rate from DKA in children is 0.15% to 0.30% [2]. Cerebral edema is a known complication of DKA and it accounts for 60% to 90% of all DKA deaths [3]. Of the cerebral oedema survivors, 10% to 25% have significant residual morbidity [2, 3].

It is known that health care costs for type 1 diabetes patients are higher than those for general population [4]. In particular, DKA is an expensive complication of type 1 diabetes. It is estimated that the mean cost per hospitalization is \$10, 876 ± 11,024 [5]. DKA was listed as the primary diagnosis for 89,000 hospital admissions and as the secondary diagnosis for 113,000 admissions [6]. In another study, it is found that the average cost for DKA hospital admission of a person (paediatric or adult) is \$13,000. This estimated cost results in a total cost of over \$1 billion annually in the United States [7].

Lack of proper management and follow-up put the children at risk of DKA and other acute complications which can be life threatening. Poor follow-up and lack of access to service increase rate of hospital admission to treat acute complications. Lack of continuity of care and point of contact after hours are important factors in increasing hospital admission to treat DKA in children and adolescents. Diabetes control relies extensively on the caregivers' day to day care. Provision of open clinic access and after hours-point of contact is crucial [8]. Instant troubleshooting is required often for children with diabetes out of hours. Such service is an important aid to prevent and to treat DKA early. Immediate and directed instructions for care giver of a child with early signs of DKA can prevent its progression and avoid the need for hospital presentation and admission.

DKA results from deficiency of circulating insulin and the combined effects of increased levels of the counterregulatory hormones: catecholamines, glucagon, cortisol, and growth hormone [9]. It is not an uncommon complication in children

and adolescents with established diabetes. Its severity can vary from mild acidosis to a life threatening condition. Mild DKA can progress to severe form if not managed properly. Accordingly, education of early signs of DKA and its management is of paramount importance to prevent and manage the condition.

Despite the improvement in diabetes management over the last decades, the frequency of DKA remained high [10]. Specialized diabetes nurse and diabetes educators are crucial members of the diabetes team. Their input is fundamental in establishing a center of excellence for quality management of diabetes in young people [11]. Training nurses in the diabetes team to be competent trainers should take a priority when setting up a diabetes center. Initial training should be followed by regular updating which requires commitment by the trainees and the employers [12].

Patient and family education is a major step in coping with diabetes management. It is proven that ensuring high family education and competence in the disease management is associated with better control, reduction of acute complication, and improvement of emotional well-being [13]. Patients should have access to a 24-hour telephone helpline managed by highly trained educators for emergency advice and treatment of DKA [14].

Experienced diabetes educators are valuable members of the diabetes team. We hypothesize that implementing a diabetes educator model of care for children and adolescents with diabetes reduces the frequency of hospital admission for DKA.

2. Materials and Methods

Information on the episodes on hospital admission for acute presentation of DKA was searched for. Data was obtained through Mafraq Hospital Health Information System (HIS) from the Medical Records Department. Search was limited to paediatric wards, medical wards, and intensive care units. Obtained data were filtered to capture number of admissions for patients 18 years old and under who have established diabetes and were admitted with the diagnosis of DKA. Venous blood pH was available in all patients included and reading below 7.3 was considered acidosis. Number and details of patients admitted were provided by the HIS Department. Data requested was for admission in the year of 2009 and 2010 (data point A). Patients who were admitted with DKA as a first sign of new diagnosis of diabetes were excluded.

The number of patients with a diagnosis of diabetes under the age of 18 and under follow-up in the outpatient clinic is also obtained (data point B). The baseline measurement was taken as a ration of number of patients admitted for DKA per total number of patients followed up in clinic A/B.

Quarterly data of patients 18 years and under who were admitted for DKA was obtained through each ward record. A study team member is allocated on 3-month basis to collect the information and data is compiled in a shared folder used by study team members. A paediatric diabetes clinic database is updated on 3-month basis to include all patients who are 18 years and under with the diagnosis of diabetes and are under regular follow-up in the Diabetes Clinic at the Paediatric Endocrinology Department, Mafraq Hospital.

A yearly ration is calculated with the numerator consisting of number of patients admitted in DKA and denominator of number of patients under regular follow-up at the diabetes clinic.

Patients with diabetes who were admitted acutely for concurrent illnesses or other indications rather than DKA were excluded. Newly diagnosed patients with diabetes presenting with DKA were also excluded. As the measure was specific to our unit performance, patients who presented and were admitted with DKA in our hospital but are not followed up in our outpatient department were excluded too.

2.1. Strategy: Setting Up the Diabetes Educator Care Model. Four nurses who are employed to cover diabetes service in the hospital received intensive training to qualify as diabetes educators. Training included provision of a high quality education in all aspects of diabetes management. Educators received special training on the use of technology in treatment of diabetes and were trained on research methodology, data management, and IT related issues through enrollment in various management and IT courses. The educators were 3 females and one male and collectively spoke 6 different languages. The paediatric diabetes case load was divided between the 4 diabetes educators. One diabetes educator was promoted as the paediatric diabetes service coordinator and was in charge of maintaining and updating the clinic database. She also has the task of allocating patients to educators based on

(i) number of patients per educator,

(ii) preferred gender of educator by family,

(iii) language spoken,

(iv) special interest of the educator in relation to treatment technology used per each patient.

Each patient was allocated a named diabetes educator to be the first line to access of service and was provided with open access to diabetes clinic. Patients were provided with direct contact number of the named educator after hours. Each educator offered 24/7 telephone access to his/her group of patients.

The role of the named educators included introduction of initial and ongoing patient education, setting up group education meetings, training patients at a high level on devices and equipment used to manage diabetes, and conducting awareness campaigns for diabetes. Patients and families were educated on the importance of patient-team collaboration to prevent and treat diabetes complication. They were, also, engaged in various special education programs and involved in departmental surveys and feedback.

Education on DKA recognition, prevention, and management received a high priority in patients and family education. Educators used various audiovisual methods to explain the condition to patients who were given written and digital material for consolidating the knowledge they

Table 1

Year	Number of admissions per year (A)	Number of patients on OPD follow-up (B)	% of admission	Ratio A/B	Fold reduction compared to baseline
2009-2010	78	37	210%	2.1	Baseline
2010-2011	41	79	51%	0.5	4.2
2011-2012	21	190	11%	0.1	21
2012-2013	12	272	4.4%	0.04	52.5
2013-2014	6	331	1.8%	0.01	210

gain from education sessions. Patients are also trained on using blood and urine ketone strips to detect early signs of DKA. Some patients had access to home blood ketone strips. Regardless of the method of ketone detection, all patients were instructed on the level at which management of ketosis should be started. Detailed instruction on how to avoid and treat DKA has given special attention to patients on insulin pump therapy.

2.2. Statistical Analysis. Chi square test is performed to test the difference in admission rate through the study period. P value is considered significant if it was found to be less than 0.05.

3. Results

3.1. Patients and Treatment Characteristics. Majority of patients followed up in clinic had type 1 diabetes. Adolescents with type 2 diabetes constituted around 5% and those with monogenic diabetes (including various types of neonatal diabetes) constituted another 5%. Over the study period, insulin pump use has increased to 50% in 2014 when half of the patients used insulin pumps and the other half was managed on multiple daily injections of insulin.

3.2. Diabetes Educators' Work Load and Seasonal Variation. Number of patients per educator increased from around 15 paediatric patients per educator at the start of study to approximately 90 patients in 2014. Diabetes educators received an average of 5 calls per week from patients. More phone calls were received from patients on insulin pumps particularly during the first few days and weeks of pump insertion. The younger the patient was, the more the phone calls were received. There was an obvious increase in phone call consultation over certain seasons of the year. Ramadan fasting month was a season when more phone calls were received.

There were 158 admissions for DKA in children and adolescents 18 years and under over a 5-year period (2009–2014). The number of admissions per year is detailed in Table 1. A total number of patients followed up in the outpatient diabetes clinics ranged between 37–331 patients from 2009 until 2014 (Table 1).

Admission rate showed a downward trend over the five-year period (Figure 1). Ratio between admission episodes

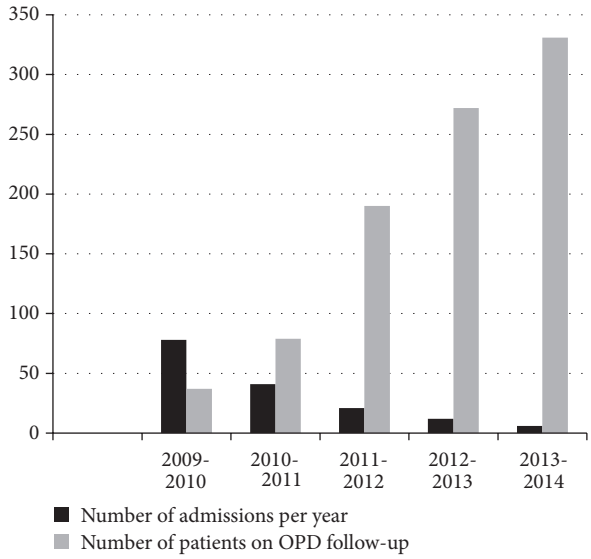

Figure 1

and outpatient case load showed a marked reduction from 2.1 in the baseline measurement year to an average of 0.16 over the next 4 years (2010–2014) with a ratio of 0.01 in the last year of the project (2014). Chi square test confirmed that the reduction in the admission rate is statistically significant with a P value of 0.001. Admission rate went from a 4.2-fold reduction for the second year after baseline measure to 210-fold reduction 4 years later (P 0.001) (Table 1).

4. Discussion

DKA is a serious complication of diabetes that can be life threatening. Medical expenditure for this potentially preventable complication is substantial and imposes a large economic burden on the healthcare system [4–7]. Despite substantial progress in overall diabetes management over the past few decades, the incidence of DKA remains high [10].

It is estimated that the risk of DKA in established type 1 diabetes patients can be up to 10% per patient per year [1]. Our study showed that implementation of our diabetes care model has reduced the percentage of DKA admission to 1.8%. Table 1 shows a steady reduction of the DKA admission per

year per 100 patients from 210, 51, 11, and 4.4 to 1.8 for years from 2009 to 2014.

DKA is considered potentially entirely preventable complication of type 1 diabetes in young people. Hospital admission because of DKA not only imposes large expenses on health care institute but disrupts the person's routine family life and leads to school and work absence. As the incidence of DKA remains high, its associated hospitalization remains high, too. Curtis et al. showed that over one-third of hospital admissions in children younger than 19 years with type 1 diabetes are due to DKA [15]. Proper education and regular follow-up reduce the possibility of this complication and its resulting hospitalization. Adapting a diabetes educator care model offers patients direct access to support and first-hand troubleshooting advice. Well trained diabetes educator can be the first on-call team members in threating emergencies and can guide patients to proper management to avoid a complication like DKA. Diabetes centers caring for children and adolescents must be equipped with highly trained diabetes educators who can provide a high quality specialized education which will empower patients and families for self-management and provide required support to manage threatening emergencies.

Many barriers are known leading to delay in diagnosing DKA. Of those, there are difficulty in distinguishing cases of DKA from other illnesses, information overload, and ling period from initial diagnosis [16]. Such barriers remain a major obstacle in prevention of DKA in children even in developed countries with active prevention campaigns [17]. Some groups of patients are known to be of high risk to develop DKA and intensive education to prevent this complication should be provided [18, 19].

Our project results proved the importance of training the trainers to deliver the required level of skills and education to the patient (end-user). It showed the importance of establishing a multidisciplinary team in treating a chronic disease like diabetes and minimizing DKA as an acute and possibly life threatening condition. Although training diabetes educators to a high level is expensive, it remains cost-effective when putting the cost of admission for DKA into consideration.

We had many limitations during execution of the project. Specialist nurses/diabetes educators are not widely available specialists. Employing nurses and training them to the level of diabetes educators incur a high cost and stretch institutions' budgets. Implementing of various patient education programs and preparing unified education material impose time and effort constraints. To accomplish an improvement in the primary end point of reduction hospital admission due to DKA, various devices and expensive ketone-detecting strips are required. These strips utilize c apillary blood samples for ketone measurement. They are accurate and can be used reliably instead of plasma or urine samples [20]. Further limitations in prevention of DKA are that some patients are not fully insured and many insurers do not cover diabetes management accessories. In search of diabetes youth study, lack of private health insurance was found to be a risk factor in development of DKA [21]. Implementing and sustaining the diabetes care model can be hard to achieve in institution with limited resources.

There were many limitations to our study. We did not have a control group to confirm effectiveness of the management model. In addition, other factors could contribute to the decrease in DKA, for example, changing the modality of treatment or the insulin regime, increased general improvement of care, and the increase in general awareness of diabetes.

In conclusion, proper guidance on prevention of DKA avoids hospital admission and saves a considerable cost and hospital resources. More importantly, it will empower children and families to self-management and boost their confidence in preventing and treating complications and emergency situation related to diabetes. Our project shows that establishing a diabetes educator care model in managing young people with diabetes is an effective and a sustainable measure to prevent hospital admission of DKA. Out of hours hotline telephone access is a major factor for preventing admission for DKA.

Additional Points

Project Summary. DKA is a serious complication of diabetes that can be life threatening. It is not an uncommon condition with an estimated risk of up to 10% per patient per year. Management of established DKA needs admission in a specialized center and imposes major constraints on hospital resources. Provision of patient education program which includes direct access of care during and after hours can reduce the risk of DKA and minimize the need for hospital admission. We have proposed a model of care led by diabetes educators for children and adolescents with diabetes. The model consists of a package of service for the patient that relies predominantly on structured education and provision of continuity of care and direct access to service. We have measured the effectiveness of the implemented model by comparing the rate of hospital admission for DKA over 4-year period compared to the baseline year prior to implementing the model. We have shown a statistically significant reduction in the admission rate for DKA during the study period. The ratio of the number of admissions to the number of patients followed up in the diabetes outpatient clinic reduced from 2.1 to 0.01 which is equivalent to 210-fold reduction. We conclude that diabetes educator care model is an effective and a sustainable measure way to reduce hospital admission for DKA.

Ethical Approval

Ethical approval was not required.

Disclosure

The study is deemed as a quality improvement project.

Competing Interests

The authors declared that there is no conflict of interests to declare.

Authors' Contributions

Asma Deeb designed the study, wrote the paper, and liaised between the team members. Hana Yousef, Layla Abdelrahman, Shaker Suliman, and Mary Tomy obtained patients' records from the admission departments and collected the prospective data throughout the study period. Salima Attia and Hana Al Suwaidi collated the data and revised the paper.

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Research Article

Risk Factors for Macro- and Microvascular Complications among Older Adults with Diagnosed Type 2 Diabetes: Findings from The Irish Longitudinal Study on Ageing

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Objective. To explore risk factors for macro- and microvascular complications in a nationally representative sample of adults aged 50 years and over with type 2 diabetes in Ireland. *Methods*. Data from the first wave of The Irish Longitudinal Study on Ageing (TILDA) (2009–2011) was used in cross-sectional analysis. The presence of doctor diagnosis of diabetes, risk factors, and macro- and microvascular complications were determined by self-report. Gender-specific differences in risk factor prevalence were assessed with the chi-squared test. Binomial regression analysis was conducted to explore independent associations between established risk factors and diabetes-related complications. *Results*. Among 8175 respondents, 655 were classified as having type 2 diabetes. Older age, being male, a history of smoking, a lower level of physical activity, and a diagnosis of high cholesterol were independent predictors of macrovascular complications. Diabetes diagnosis of 10 or more years, a history of smoking, and a diagnosis of hypertension were associated with an increased risk of microvascular complications. Older age, third-level education, and a high level of physical activity were protective factors ($p < 0.05$). *Conclusions*. Early intervention to target modifiable risk factors is urgently needed to reduce diabetes-related morbidity in the older population in Ireland.

1. Introduction

Over the past two decades, the global burden of diabetes has increased significantly [1–3]. Between 1998 and 2015, the prevalence of diabetes increased from 2.2% to 5.2% among the adult population in Ireland [4]. In 2010, diabetes was the ninth leading cause of mortality [1] and the 14th largest cause of disability adjusted life years (DALYs) [2] worldwide. The economic cost of diabetes is also high and will continue to rise; approximately 12% of the world's total health expenditure was spent on diabetes in 2010 [5]. The vast majority of this burden is attributable to the macrovascular (myocardial infarction, congestive cardiac failure, and cerebrovascular accident) and microvascular (diabetic neuropathy, retinopathy, and nephropathy) complications of diabetes [3]. The Cost of Diabetes in Europe-Type II study reported that 72% of people had at least one diabetes-related complication [6]. In Ireland, the prevalence of macro- and microvascular complications among adults aged 50 years and over with type 2 diabetes is 15% and 26%, respectively [7].

Compared to the general population, individuals with type 2 diabetes are at an increased risk of developing cardiovascular disease (CVD) [8, 9]. Type 2 diabetes is also a significant cause of blindness in adults, nontraumatic lower limb amputations, and end-stage renal disease resulting in transplantation and dialysis [3]. We reported that the risk of visual impairment, in adults aged 50–69 years, is approximately four times higher in the population with

diabetes compared to those without diabetes [10]. Buckley et al. [11] found that the risk of an individual with diabetes undergoing lower-leg amputation was 22 times that of an individual without diabetes.

Time-related variables, such as age and longer diabetes duration, are associated with the onset of diabetes-related complications [12]. Established risk factors for CVD such as hypertension, high cholesterol, and smoking further increase the likelihood of developing both macro- and microvascular complications [13]. Socioeconomic status (SES) has also been identified as a predictor of microvascular complications. Lower educational attainment is considered to influence the development of such complications through health behaviours, access to care, and processes of diabetes care [14–17]. While the national prevalence of diagnosed type 2 diabetes and related complications has been established among the older population in Ireland [7], evidence on the individual level risk factors for macro- and microvascular complications is lacking. Identification and treatment of risk factors can delay or prevent the development of diabetes-related complications [3]. Therefore, the purpose of this paper is to identify the determinants of macro- and microvascular complications among adults aged 50 years and over with diagnosed type 2 diabetes.

2. Material and Methods

2.1. Data Source. Data from the first wave (2009–2011) of The Irish Longitudinal Study on Ageing (TILDA), a population-based prospective cohort study of community-dwelling adults aged 50 and over in Ireland, were used in this cross-sectional analysis [18]. A nationally representative sample was selected using the RANSAM sampling technique from a listing of all residential addresses in Ireland (the Irish Geodirectory) [19]. A total of 8175 adults aged 50 years and over completed a computer-assisted personal interview (CAPI), representing a household response rate of 62%. The CAPI was administered by trained social interviewers in the participants' homes. This recorded detailed information on health, social, and economic circumstances. During the CAPI, participants reported their medication use and interviewers noted the correct name from the medication packaging. Medications were assigned World Health Organization Anatomic Therapeutic Chemical (ATC) classification codes [20]. Ethical approval was obtained from the Faculty of Health Sciences Research Ethics Committee of Trinity College Dublin. Written informed consent was provided by all respondents before participation [18].

2.2. Type 2 Diabetes Classification. Methods to classify diagnosed type 2 diabetes and macro- and microvascular complications have been reported in detail elsewhere [7]. In brief, individuals were classified as having diagnosed diabetes if they self-reported a previous doctor diagnosis or if they reported the use of insulin or oral hypoglycaemic agents during the CAPI. Age at diabetes diagnosis (years) was established by self-report. Anyone aged less than 40 years at diagnosis and injecting insulin but not on oral hypoglycaemic agents was classified as having type 1 diabetes; all others were classified as having diagnosed type 2 diabetes [21].

2.3. Macrovascular and Microvascular Complications. All TILDA participants were asked the question "*Has a doctor ever told you that you have any of the conditions on this card?*" Heart attack (myocardial infarction), heart failure (congestive cardiac failure), stroke (cerebrovascular accident), and mini stroke (transient ischemic attack (TIA)) were included in the list and were defined as macrovascular complications. Participants who reported a doctor diagnosis of diabetes were asked the question "*Has a doctor ever told you that you have any of the following conditions related to your diabetes?*" The conditions listed were leg ulcer, protein in urine (proteinuria), lack of feeling and tingling pain in legs and feet due to nerve damage (diabetic neuropathy), damage to the back of your eye (diabetic retinopathy), or damage to your kidneys (diabetic nephropathy); these were defined as microvascular complications [21]. Macro- and microvascular complications were collapsed into dichotomous variables, to indicate the presence or absence of at least one complication.

2.4. Covariates. Sociodemographic, behavioural, and medical history variables were recorded in the CAPI including sex, age (50–64 years, 65–74 years, and 75+ years), location of household (capital city (Dublin), another town/city, and rural), educational attainment (primary-level or none, secondary-level, and third-level or higher), and medical care cover (means-tested public health insurance scheme for those on low incomes, supplementary private health insurance, dual cover (both state-assisted cover and private insurance), and no additional cover for healthcare). Smoking status was classified as having ever smoked (current and former smoker) and never smoked (nonsmoker). Physical activity (low, moderate, and high) was self-reported using the short version of the International Physical Activity Questionnaire (IPAQ) and categorised using the IPAQ scoring protocol [22]. Duration of diabetes diagnosis was calculated by subtracting age at diagnosis from age (years) at interview and was subsequently categorised (0–4 years, 5–9 years, and ≥10 years). The use of diabetes medication (oral hypoglycaemic agents, insulin, or none) was reclassified as diet alone, oral agents alone, insulin alone, or oral agents and insulin. A previous doctor diagnosis of hypertension or high cholesterol was ascertained by self-report.

2.5. Statistical Analysis. Analysis was carried out in Stata version 13 for Windows (StataCorp, College Station, TX) using the survey function (svy). Inverse probability weights were applied to all analyses to provide population estimates. Weights were calculated according to the distribution of marital status, educational attainment, and geographic location using Irish census data from the period of 2006–2010 and to the distribution of age and sex using Irish census data from 2011 [23].

Descriptive statistics were used to summarise characteristics of the population with diagnosed type 2 diabetes and were stratified by gender. Gender-specific differences

in categorical variables were analysed using Pearson's chi-square test. The mean and standard deviation were reported if continuous data conformed to normality and Student's t-test was conducted to compare means. If data were skewed, the median with associated lower and upper quartile values was reported and the Mann-Whitney test was utilised.

Associations between risk factors and diabetes-related complications were examined using a log-binomial regression. Risk ratios (RR) and 95% CI were generated as a measure of association. Risk factors served as independent variables and were chosen on the basis of previous literature [12, 14, 24–35] or if significant associations were observed in univariate analysis. A forward blockwise entry method was used with independent variables which were entered into the regression model in three blocks: (1) sociodemographic variables (age, sex, and education attainment) and duration of diabetes diagnosis, (2) behavioural factors (ever smoking and level of physical activity), and (3) medical history variables (diagnosis of hypertension, high cholesterol). Collinearity was assessed by the variance inflation factor (VIF); a VIF of >10 indicated multicollinearity. Statistical significance was defined as $p < 0.05$.

3. Results

Of the 8175 participants in TILDA, 634 participants reported a previous doctor diagnosis of diabetes and 38 participants did not report a diagnosis of diabetes but were classified as having diabetes by the use of oral hypoglycaemic agents or insulin. Of these, 17 people were classified as having type 1 diabetes and were excluded from the present analysis. Of the 655 people with type 2 diabetes, 57.7% were male and the mean age was 66.6 (SD = 8.8) years. Table 1 shows the characteristics of the population with type 2 diabetes, stratified by gender. Approximately half of the participants were diagnosed with diabetes between the ages of 50–69 years (51.8% [47.6% to 55.9%]). The median time since diabetes diagnosis was 5.3 years (IQR 2 to 11 years). Approximately half the study sample had completed secondary- or third-level education (51.1% [46.9% to 55.4%]). In terms of disease management, 17.5% (14.8% to 20.6%) of the participants managed type 2 diabetes with diet alone, 74.2% (70.6% to 77.5%) reported the use of oral hypoglycaemic agents, and 7.5% (5.6% to 10.0%) reported using both oral hypoglycaemic agents and insulin. The prevalence of cardiovascular risk factors was high among participants; 18.8% (15.8% to 22.3%) of the participants were current smokers, 62.5% (58.1% to 66.6%) reported a previous doctor diagnosis of hypertension, and 51.7% (47.5% to 55.9) reported a previous doctor diagnosis of high cholesterol. Current smoking was higher among females compared to males (20.6% [15.5% to 26.5%] versus 17.5% [13.9% to 26.5%]) and a higher proportion of males reported higher levels of physical activity (27.5% [23.2% to 32.3%] versus 13.7% [9.6% to 19.2%]). Fifteen percent (15.1% [12.3% to 18.4%]) of the participants reported a previous doctor diagnosis of at least one macrovascular complication and 26% (25.5% to 29.9%) of the participants reported a previous doctor diagnosis of at least one microvascular complication.

3.1. Factors Associated with Diabetes-Related Complications. Table 2 presents the results from the binomial regression analyses, where previous diagnosis of at least one macrovascular complication served as the dependant variable. The risk of a macrovascular complication was higher among older participants (RR 1.6 [1.1 to 2.5] in 65 to 74 years; RR 2.0 [1.2 to 3.2] in 75 years or over versus 50 to 64 years). Female participants were less likely to report a previous diagnosis of a macrovascular complication relative to males (RR 0.6 [0.4–0.8]). Finally, individuals classified as having ever smoked had a 60% increase in the risk of a macrovascular complication compared to those who never smoked (RR 1.6 [1.1 to 2.6]).

Table 3 presents the results from the binomial regression analyses, with previous diagnosis of at least one microvascular complication as the dependant variable. There was no evidence to suggest that the risk of microvascular complications was different in participants diagnosed for less than four years compared to those diagnosed for a period between five and nine years (RR 1.1 [0.8 to 1.7]), whereas participants with type 2 diabetes for 10 or more years were approximately twice as likely to have reported a microvascular complication compared to those who had been diagnosed for less than four years (RR 1.9 [1.4 to 2.5]). Participants who had ever smoked were almost one and a half times more likely to report a doctor diagnosis of a microvascular complication relative to those who never smoked (RR 1.4 [1.1 to 2.0]). While the risk of a microvascular complication did not differ between those with a secondary-level education compared to those with primary-level or less education (RR 0.9 [0.6 to 1.2]), the risk was significantly decreased in participants with a third-level education (RR 0.6 [0.4 to 0.9]) compared to those with primary-level or less education. The risk of a microvascular complication did not differ between participants who reported a moderate level of physical activity compared to those with low level of activity (RR 0.8 [0.6 to 1.1]). However, participants from the highest physical activity category were 50% less likely to report a microvascular complication (RR 0.5 [0.3–0.8]) compared to participants with low level of activity. Participants diagnosed with hypertension were one and a half times more likely to have reported a previous diagnosis of at least one microvascular complication relative to participants who had not reported a previous diagnosis (RR 1.5 [1.1–2.1]). Collinearity was not found between variables (VIF ≤ 2).

4. Discussion

To our knowledge, this is the first study to identify individual level risk factors associated with macrovascular and microvascular complications among people with type 2 diabetes in Ireland using nationally representative data. Older age, having ever smoked, and a previous doctor diagnosis of high cholesterol were independently associated with an increased risk of macrovascular complications, while being female and high levels of physical activity demonstrated a protective effect. Longer duration since diagnosis, having ever smoked, and a previous doctor diagnosis of hypertension were associated with an increased risk of microvascular complications whereas achieving a third-level or higher education

Table 1: Descriptive characteristics of the first-wave TILDA sample aged ≥50 years with diagnosed type 2 diabetes, 2009–2011.

Variable	Type 2 diabetes (total n = 655)			
	Male n (%)	Female n (%)	p	Total % (95% CI)
Age				
Years (mean, sd.)	66.6 (9.7)	68.6 (10.4)		67.4 (10.1)
Location				
Dublin	92 (25.5)	77 (31.5)	0.17	27.9 (23.1, 33.4)
Another city	113 (30.0)	84 (34.1)		31.7 (26.9, 36.9)
Rural	182 (44.5)	105 (35.3)		40.3 (35.2, 45.7)
Educational attainment				
Primary/none	163 (48.6)	116 (54.7)	0.12	51.1 (46.9, 55.4)
Secondary	137 (37.1)	103 (36.1)		36.7 (32.9, 40.7)
Third-level/higher	89 (14.3)	47 (9.2)		12.2 (10.1, 14.6)
Medical cover				
State-assisted	163 (43.9)	139 (57.8)	<0.01	49.7 (45.4, 53.9)
Private insurance	111 (27.6)	60 (17.9)		23.6 (20.3, 27.2)
Dual cover	86 (19.9)	51 (18.7)		19.4 (16.2, 23.0)
No additional cover	30 (8.5)	16 (5.6)		7.3 (5.3, 10.1)
Smoking				
Never	114 (29.9)	120 (44.0)	<0.001	35.8 (31.8, 39.9)
Past	210 (52.7)	94 (35.4)		45.5 (41.2, 49.9)
Current	66 (17.5)	52 (20.1)		18.8 (15.8, 22.3)
Physical activity				
Low	140 (36.8)	143 (56.5)	<0.001	45.0 (40.8, 49.2)
Moderate	137 (35.7)	77 (29.8)		33.3 (29.5, 37.2)
High	109 (27.5)	43 (13.7)		21.8 (18.5, 25.5)
Age of diagnosis				
<50 years	54 (16.4)	47 (16.8)	0.04	16.6 (13.7, 19.8)
50–69 years	196 (55.9)	125 (46.2)		51.8 (47.6, 55.9)
70+ years	112 (27.7)	84 (36.9)		31.6 (27.8, 35.7)
Duration of DM diagnosis				
Years (median, IQR)	5.0 (1.5, 12)	5.5 (2.5, 15)	0.5	5.3 (2, 11)
DM treatment				
Diet	64 (16.7)	53 (18.3)	0.9	17.5 (14.8, 20.6)
Oral meds	293 (74.7)	190 (73.9)		74.2 (70.6, 77.5)
Insulin	2 (1.0)	4 (0.8)		0.8 (0.3, 1.8)
Both	31 (8.2)	19 (6.5)		7.5 (5.6, 10.0)
Other medication				
Hypertension	221 (58.4)	161 (60.0)	0.6	59.1 (55.4, 63.9)
High cholesterol	164 (43.4)	130 (46.5)	0.4	44.7 (40.4, 48.7)
Flu shot	286 (74.3)	212 (78.5)	0.2	76.1 (72.4, 79.5)
Doctor diagnosed				
Hypertension	236 (61.2)	170 (63.8)	0.9	62.5 (58.1, 66.6)
High cholesterol	192 (50.0)	149 (54.2)	0.9	51.7 (47.5, 55.9)
DM complications				
Macro	73 (17.8)	28 (11.4)	0.04	15.1 (12.3, 18.4)
Micro	89 (25.2)	67 (26.8)	0.7	26.0 (22.5, 29.9)

Table 2: Multivariate binomial regression models exploring independent associations between macrovascular complications and predictor variables*.

Predictor	Model 1†		Model 2‡		Model 3§	
	RR (95% CI)	p	RR (95% CI)	p	RR (95% CI)	p
Age						
50–64 years	1		1		1	
65–74 years	1.6 (1.0, 2.5)	0.05	1.5 (0.9, 2.4)	0.07	1.6 (1.1, 2.5)	0.04
75+ years	2.0 (1.3, 3.3)	0.003	1.8 (1.1, 4.1)	0.01	2.0 (1.2, 3.2)	0.005
Gender						
Male	1		1		1	
Female	0.6 (04, 0.9)	0.007	0.6 (0.4, 0.9)	0.009	0.6 (0.4, 0.8)	0.005
Education						
Primary/less	1		1		1	
Secondary	0.9 (0.6, 1.3)	0.5	0.9 (0.6, 1.4)	0.5	0.9 (0.6, 1.3)	0.6
Third/higher	1.1 (0.7, 1.7)	0.7	1.2 (0.7, 1.8)	0.5	1.1 (0.7, 1.7)	0.7
Duration of diagnosis						
0–4 years	1		1		1	
5–9 years	1.0 (0.6, 1.7)	0.9	0.9 (0.6, 1.6)	0.9	1.0 (0.6, 1.7)	0.9
10+ years	1.2 (0.8, 1.8)	0.4	1.1 (0.7, 1.6)	0.7	1.1 (0.8, 1.7)	0.5
Ever smoked						
No			1		1	
Yes			1.7 (1.1, 2.7)	0.02	1.6 (1.1, 2.6)	0.04
Physical activity						
Low			1		1	
Medium			0.8 (0.6, 1.2)	0.3	0.9 (0.6, 1.2)	0.5
High			0.5 (0.3, 0.9)	0.01	0.5 (0.3, 0.9)	0.03
Previous hypertension						
No					1	
Yes					1.1 (0.8, 1.7)	0.5
Previous high cholesterol						
No					1	
Yes					1.7 (1.1, 2.5)	0.008

*Indicating report of at least one macrovascular complication (heart attack, congestive heart failure, stroke, or TIA).

†Variables entered in Model 1: age, sex, education, and years since diagnosis.

‡Variables entered in Model 2: age, sex, education, years since diagnosis, smoking status, and physical activity.

§Variables entered in Model 3: age, sex, education, years since diagnosis, smoking status, physical activity, doctor diagnosed hypertension, and doctor diagnosed high cholesterol.

and high levels of physical activity demonstrated a protective effect.

Consistent with existing research [28, 29], established risk factors for CVD [28, 29] and being male [28, 36] were found to be independent predictors of at least one macrovascular complication. The United Kingdom Prospective Diabetes Study (UKPDS) reported that the risk of myocardial infarction and stroke were higher in older participants, smokers, and those with high cholesterol [28, 29]. Prospective studies have also identified hypertension [28–30, 32] as a risk factor for the development of macrovascular complications among individuals with type 2 diabetes; however, our study failed to demonstrate a significant association. Our findings are not atypical of existing research in this area; the diversity of results has been discussed previously [28–30, 37]. Evidence demonstrating an association between duration of diabetes diagnosis and macrovascular complications is also equivocal. Some studies are in accordance with our findings [37] whereas others have reported the opposite [12, 29]. Similar to the present study, Fox et al. [37] failed to demonstrate duration of diabetes as an independent predictor of combined nonfatal macrovascular events (myocardial infarction, stroke, congestive heart failure, and angina) [37], whereas baseline data from the ADVANCE trial [12] demonstrated that diabetes duration was an independent predictor of nonfatal myocardial infarction and nonfatal stroke among 11,140 individuals with type 2 diabetes aged 55 years and older. Unlike the previous study [12], we were unable to conduct complication-specific analysis due to the small number of reported events.

Consistent with previous research [12, 24–26, 33, 35], longer duration since diabetes diagnosis was independently associated with microvascular complications. Diabetes duration reflects total glycaemic control and risk factor exposure

Table 3: Multivariate binomial regression models exploring independent associations between microvascular complications and predictor variables*.

Predictor	Model 1†		Model 2‡		Model 3§	
	RR (95% CI)	p	RR (95% CI)	p	RR (95% CI)	p
Age						
50–64 years	1		1		1	
65–74 years	0.8 (0.6, 1.2)	0.4	0.9 (0.7, 1.2)	0.4	0.9 (0.7, 1.1)	0.3
75+ years	0.7 (0.5, 1.1)	0.1	0.7 (0.5, 0.9)	0.02	0.6 (0.5, 0.9)	0.01
Gender						
Male	1		1		1	
Female	1.0 (0.8, 1.4)	0.9	1.1 (0.7, 1.3)	0.8	1.0 (0.8, 1.3)	0.9
Education						
Primary/less	1		1		1	
Secondary	0.9 (0.7, 1.2)	0.4	0.9 (0.7, 1.2)	0.5	0.9 (0.7, 1.2)	0.5
Third/higher	0.6 (0.4, 0.8)	0.007	0.5 (0.4, 0.9)	0.02	0.6 (0.4, 0.9)	0.02
Duration of diagnosis						
0–4 years	1		1		1	
5–9 years	1.2 (0.8, 1.8)	0.3	1.1 (0.8, 1.6)	0.5	1.1 (0.8, 1.7)	0.5
10+ years	2.0 (1.5, 2.7)	0.000	1.8 (1.4, 2.5)	0.000	1.9 (1.4, 2.5)	0.000
Ever smoked						
No			1		1	
Yes			1.4 (1.1, 2.0)	0.03	1.4 (1.1, 2.0)	0.02
Physical activity						
Low			1		1	
Medium			0.7 (0.6, 1.0)	0.1	0.8 (0.6, 1.1)	0.1
High			0.5 (0.3, 0.7)	0.001	0.5 (0.3, 0.8)	0.003
Hypertension						
No					1	
Yes					1.5 (1.1, 2.1)	0.006
High cholesterol						
No					1	
Yes					1.0 (0.8, 1.3)	0.9

*Indicating report of at least one microvascular complication (leg ulcer, protein in urine, neuropathy, retinopathy, or damage to kidneys).

†Variables entered in Model 1: age, sex, education, and years since diagnosis.

‡Variables entered in Model 2: age, sex, education, years since diagnosis, smoking status, and physical activity.

§Variables entered in Model 3: age, sex, education, years since diagnosis, smoking status, physical activity, doctor diagnosed hypertension, and doctor diagnosed high cholesterol.

over time [38]. Likewise, both smoking and hypertension have been identified as prominent risk factors in the development of neuropathy, retinopathy, and nephropathy [24–26, 30, 31, 39, 40]. Similar to previous findings [14, 24, 35, 41–43], higher educational attainment was associated with a lower likelihood of microvascular complications in the present study. Education is a universal indicator of SES and is commonly used in cardiovascular epidemiology as it usually remains constant after early childhood and is less likely to be influenced by social changes or illness in adulthood [44]. Lower educational attainment has been associated with poorer disease management, lower rates of physical activity, fewer ophthalmologic visits, and fewer foot examinations [14]. Earlier detection by systematic screening can prevent or delay the development of diabetes-related complications. Reductions in leg amputation rates have been achieved in the UK following changes to the structure of foot care for those with diabetes [45, 46].

In the present study, the risk of microvascular complications was lower in participants who reported a high level of physical activity compared to the lowest physical activity group. This protective effect on microvascular morbidity has been highlighted previously [25]. High levels of physical activity are beneficial for individuals with type 2 diabetes as it is linked with better glucose control [25]. Similar to the present study, the Health and Retirement Study (HRS) in the USA [34] reported that individuals with diabetes-related microvascular complications were less likely to engage in high levels of physical activity. Janevic et al. [34] suggest that the development of diabetes-related complications may cause clinical, practical, and psychological barriers to engaging in physical activity. Therefore, additional support may be

needed to achieve the recommended amounts of physical activity in those who have developed complications [34].

The major strength of this study is the large national population-based sample and the high response rate (62%). Inverse probability weights were calculated to take into account the underrepresentation of individuals with lower levels of education attainment and to adjust for the lower response rate in age and sex groupings [20]. Study weights were applied to all analyses to correct for differential nonresponse. Therefore, selection bias was minimised and TILDA sample is representative of the general Irish population [20].

However, several limitations need to be considered when interpreting the findings. Firstly, data used in the analyses were based on self-report and were not ascertained by an objective method. Self-reporting is a recognised limitation in all surveys due to potential inaccuracies and recall and reporting bias [47]. When compared with medical records, data based on self-report have been shown to underestimate the prevalence of diabetic retinopathy [48] and heart failure [49]. In the present study, the prevalence of complications may have been underestimated; as a consequence the measure of association may be biased toward the null. However, moderate-to-high levels of agreement, between self-report and medical records, have been demonstrated for diabetes [49, 50], myocardial infarction [49], stroke [49, 50], and hypertension [49]. Data on smoking and physical activity were also based on self-report where socially desirable responses are a documented phenomenon [47].

Secondly, recall bias should be considered. Participants who were recently diagnosed with diabetes may remember the age of their diagnosis with greater precision. Incorrect reporting may result in differential misclassification and could lead to a subsequent decrease in the measure of association if the number of years since diabetes diagnosis has been overestimated. Nevertheless, the previously documented association between microvascular disease and longer duration since diagnosis [12, 24–26, 33, 35] was detected in this study. Finally, a cross-sectional study design does not permit assessment of causality. For instance, it is not possible to infer if a high level of physical activity reduces the risk of microvascular complications or whether the development of microvascular complications inhibits physical activity [34].

5. Conclusions

Despite these limitations, findings from this study are in accordance with other research from prospective cohort studies. We demonstrated that macrovascular complications were more common in the male population and the probability of microvascular complications was reduced in participants with higher educational attainment. Additionally, modifiable risk factors were independently associated with both macro- and microvascular complications. While addressing lifestyle factors is a key part of preventing complications, delivering adequate services for people with diabetes is essential in earlier detection and management of complications. In 2010, a national diabetes programme was introduced in Ireland [51]. To date, the programme has been instrumental in the rollout of a national retinal screening programme, the recruitment of diabetes nurse specialists, and development of a national foot care model [51]. Macrovascular and microvascular complications are often preventable; therefore findings from this study are useful for policy makers planning the development of other diabetes services, including the diabetes cycle of care that has been recently introduced into primary care in Ireland [52]. Diabetes prevalence is projected to increase; therefore effective prevention strategies are urgently needed to reduce the future burden of complications in Ireland.

Competing Interests

No competing interests were known or perceived.

Authors' Contributions

Marsha L. Tracey and Patricia M. Kearney conceived and designed the study. Marsha L. Tracey researched data. Marsha L. Tracey analysed the data. Marsha L. Tracey wrote the paper. Patricia M. Kearney, Sheena M. McHugh, Anthony P. Fitzgerald, Claire M. Buckley, and Ronan J. Canavan reviewed the paper. Marsha L. Tracey edited the paper. Marsha L. Tracey, Patricia M. Kearney, Sheena M. McHugh, Anthony P. Fitzgerald, Claire M. Buckley, and Ronan J. Canavan approved the final paper.

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Research Article

Influence of Age at Diagnosis and Time-Dependent Risk Factors on the Development of Diabetic Retinopathy in Patients with Type 1 Diabetes

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Aim. To determine the influence of age at onset of type 1 diabetes and of traditional vascular risk factors on the development of diabetic retinopathy, in a cohort of patients who have been followed up after onset. *Methods*. Observational, retrospective study. The cohort consists of 989 patients who were followed up after diagnosis for a mean of 10.1 (SD: 6.8) years. The influence of age at diagnosis, glycemic control, duration of diabetes, sex, blood pressure, lipids, BMI, and smoking is analyzed using Cox univariate and multivariate models with fixed and time-dependent variables. *Results*. 135 patients (13.7%) developed diabetic retinopathy. The cumulative incidence was 0.7, 5.9, and 21.8% at 5-, 10-, and 15-year follow-up, respectively. Compared to the group with onset at age <10 years, the risk of retinopathy increased 2.5-, 3-, 3.3-, and 3.7-fold in the groups with onset at 10–14, 15–29, 30–44, and >44 years, respectively. During follow-up we also observed an association between diabetic retinopathy and HbA1c levels, HDL-cholesterol, and diastolic blood pressure. *Conclusion*. The rate of diabetic retinopathy is higher in patients who were older at type 1 diabetes diagnosis. In addition, we confirmed the influence of glycemic control, HDL-cholesterol, and diastolic blood pressure on the occurrence of retinopathy.

1. Introduction

Diabetic retinopathy (DR) remains a common complication in people with type 1 diabetes (T1D). 39, 55, and 84% of T1D patients will develop DR after 10, 20, and 40 years of evolution, respectively [1–3]. The duration of diabetes and the glycemic control are the risk factors that are most closely related with all forms of DR. Other factors such as male gender, hypertension, high body mass index (BMI), nephropathy, dyslipidemia, smoking, and genetic factors appear to influence the onset or progression of DR, although their role is controversial [1, 3–5].

The influence of age at T1D diagnosis on the occurrence of microvascular complications like DR is currently a subject of active debate. Some studies have reported that the prepubertal stage, especially the first 5 years of life, might protect from the occurrence of DR [6–8]. However, there are authors that point out that such protection disappears as the disease progresses [2, 5], while others have never observed such an effect [9, 10].

Puberty has a negative influence on the appearance of DR, which is due to the combination of hormonal changes and the poorer control that often accompanies this stage of life [11]. When T1D onset is after age 15, the literature on the effect of age on DR development is limited and rather confusing. According to Hammes et al. [3], patients aged over 15 at onset have the lowest protection against advanced DR, whereas Hietala et al. [4] stated that the risk of proliferative

DR (PDR) is higher when T1D onset is between 5 and 14 than when it is between 15 and 40. Finally, Kullberg et al. [12] reported that the prevalence of DR increases in patients aged 15 to 19 at onset but decreases at onset ages between 30 and 35.

In this study we take advantage of the cohort of patients included in the Type 1 Diabetes Registry of Navarra to estimate the risk of DR development according to age at onset and duration of T1D, smoking, blood pressure (BP), BMI, glycemic control as estimated by HbA1c levels, and lipid profile.

2. Methods

This is an observational retrospective follow-up study. The subjects of the study are all included in the T1D Registry of Navarra: patients with onset of T1D from January 1990, who were followed up and treated in the "Complejo Hospitalario de Navarra" until July 2013. The cohort included 989 patients. The study protocols were approved by the regional Ethical Review Board of Navarra.

T1D was diagnosed according to clinical criteria as recommended by the World Health Organization [13]. The clinical diagnostic criteria are those previously validated by Molbak et al. [14]. In all cases, we also measured anti-GAD and anti-IA2 antibodies. According to the medical protocol followed, all patients had at least one scheduled outpatient appointment per year. The patients' data needed for the study were obtained from the electronic health records of the Navarra Health Service. We gathered information about age and sex at onset for all patients. At the screening visit and for every visit through follow-up, we included weight, height, systolic BP (SBP), diastolic BP (DBP), smoking status, and analytical data such as lipid profile and HbA1c. When patients had more than one determination of these covariates in a year, we computed the arithmetic mean in the case of continuous variables, while for the categorical ones we chose the value that lasted longest in that year.

In all follow-up visits, smoking habits were ascertained and patients were categorized as nonsmokers, ex-smokers, or smokers. A nonsmoker was defined as someone who had smoked fewer than 100 cigarettes in their lifetime; an ex-smoker was someone who had smoked more than that amount but had quit smoking at least one year before the analysis of data was performed; finally, a smoker was someone who had not quit smoking or had quit within the last year.

BP was measured once, after a rest of at least ten minutes.

BMI was calculated using the formula: weight (in kilograms) divided by height (in meters) squared.

Screening and grading for the presence or absence of DR were performed by trained ophthalmologists using fundoscopy in mydriasis at least once every two years, starting five years after diagnosis. In our hospital, T1D patients are always explored by an ophthalmologist. Retinal examination by binocular biomicroscopy and a 78/90 D lens was recorded in a standardized format in the electronic health record of the Navarra Health Service. In patients under 12 years, the standard exploration consisted of indirect ophthalmoscopy with a 20/28 D lens. Retinopathy was graded according to a 5-degree severity scale based on the American Academy of Ophthalmology's simplified classification [15]. However, since we had rather few cases of PDR, all grades were grouped together for statistical purposes.

From 1990 to 1997, HbA1c was measured using various techniques (Abbott IMX, Ciba Corning Glycomet, Merck, and Menarini HPLC), but after 1997, HbA1c was determined in all patients with high-performance liquid chromatography (HPLC; Adams A1c HA, Menarini Diagnostics, Florence, Italy; reference range: 4.1–6.2%). In 2005, the Hospital Complex obtained level II laboratory certification of traceability from the Diabetes Control and Complications Trial (DCCT) reference method through the National Glycohemoglobin Standardization Program. Previous HbA1c determinations had also been standardized to the DCCT reference range [16].

HDL-cholesterol and triglycerides (TG) were measured by GPO-PAP (Roche Diagnostics). LDL-cholesterol was calculated by the Friedewald equation.

Statistical Analysis. Characteristics of the patients at onset of the disease were summarized using frequency and percentages for categorical variables and mean and standard deviations (SD) for continuous ones. The cumulative incidence of retinopathy was estimated and graphed for the whole sample and also divided by age groups. 95% confidence intervals based on the cumulative hazard were estimated for 5, 10, and 15 years after onset. Data were right-censored when no retinopathy event occurred during follow-up or due to loss to follow-up or death.

In order to assess the effect of the different variables on retinopathy, firstly, univariate Cox-proportional hazards regression models were fitted. We assessed the effect of covariates at onset as fixed effects and complemented the analyses with a dynamic approach that includes the covariates as time-dependent variables, updating the values along the follow-up. The proportionality assumption implicit in the Cox models was assessed using weighted residuals and when violated, an interaction term with time was evaluated. The possible modifying effect of age group was evaluated and models were adjusted by age group when appropriate. Secondly, a multivariate regression model was fitted with the covariates that had turned out to be significant in the previous step.

All analyses were performed using the R statistical package, version 3.1.1.

3. Results

989 patients with T1D were followed up from onset, with mean (SD) follow-up of 10.1 (6.8) years. Of them, 292 (29.5%) had the onset in childhood (under 15 years) and 579 (58%) were men. At onset, 8 (0.8%) had lipid lowering treatment and 12 (1.2%) antihypertensive treatment, figures that increased to 143 (14.5%) and 103 (10.4%), respectively, at follow-up. Antihyperlipidaemic and antihypertensive treatment were more frequent in patients with than without retinopathy (20.7% versus 13.5% and 21.5% versus 8.7%, resp.).

Table 1: Demographic and clinical characteristics at onset of our population of T1D patients.

Variable		Total
Categorical variables		*n* (%)
Sex		
Men		579 (58%)
Women		410 (42%)
Age group (years)		
0–9		190 (19%)
10–14		192 (19%)
15–29		334 (34%)
30–44		204 (21%)
≥45		69 (7%)
Smoking		
No		613 (68%)
Ex-smoker		42 (5%)
Yes		249 (27%)
Antihypertensive treatment		
No		981 (99.2%)
Yes		8 (0.8%)
Lipid lowering treatment		
No		977 (98.8%)
Yes		12 (1.2%)
Continuous variables	*n* = 989	Mean (SD)
Years of follow-up	989	10.1 (6.8)
SBP (mmHg)	816	115.7 (14.8)
DBP (mmHg)	816	70.1 (11.4)
LDL (mg/dL)	783	116.5 (40.3)
HDL (mg/dL)	804	48.7 (15.2)
Triglycerides (mg/dL)	840	119.9 (150.9)
BMI	821	20.2 (4.4)
HbA1c (%)	793	11.0 (2.5)

SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoproteins; HDL, high density lipoproteins; BMI, body mass index; HbA1c, glycated hemoglobin.

Table 2: Cumulative incidence (IC 95%) after 5, 10, and 15 years since onset.

	Cumulative incidence of DR		
	Time since onset		
	5 years (CI)	10 years (CI)	15 years (CI)
Overall T1D population	0.7 (0.1, 1.3)	5.9 (4.0, 7.7)	21.8 (17.7, 25.7)
T1D groups by age at onset			
0–9 years	0	2.0 (0, 4.8)	3.7 (0, 7.9)
10–14 years	0	4.2 (0.5, 7.7)	18.3 (9.4, 26.3)
15–29 years	0.8 (0, 1.8)	6.5 (3.3, 9.7)	25.8 (18.8, 32.2)
30–44 years	1.7 (0, 3.6)	10.0 (4.3, 15.3)	30.3 (18.1, 40.7)
≥45 years	1.8 (0, 5.1)	7.3 (0, 15.1)	44.5 (14.7, 63.9)

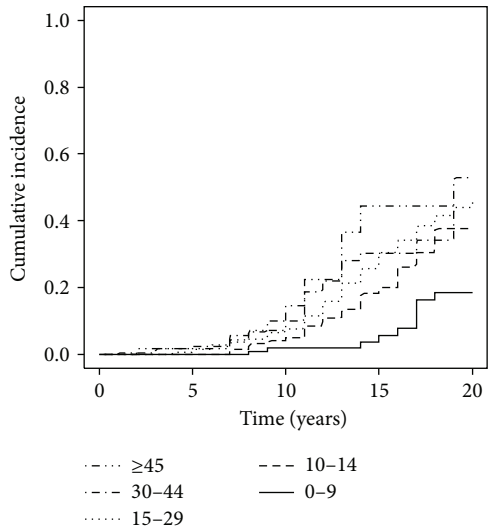

Figure 1: Cumulative incidence of retinopathy by age group.

Demographic and clinical characteristics are shown in Table 1.

At follow-up, 135 patients developed retinopathy (13.7%), 121 of whom had nonproliferative retinopathy (NPDR) and 14 PDR. All patients with PDR had been previously diagnosed with NPDR. Given the low number of patients with PDR, statistical results are focused on total retinopathy. Nevertheless, it deserves to be mentioned that, at onset, patients that develop PDR have similar HbA1c mean values compared to the rest of the patients (10.9 (4.1) versus 11.0 (2.5)), but at follow-up, patients that developed PDR had HbA1c mean values of 9.39 (2.01), whereas those that developed NPDR had 8.30 (1.51), and those with no retinopathy had 7.74 (1.33).

As expected, the cumulative incidence increased over time during the course of diabetes. It was very low during the first 5 years after onset, but it undoubtedly increased after 10 and, especially, 15 years. We observed that the rate of retinopathy was higher in patients that were older at diagnosis. The highest increase was observed in the group whose onset was at age ≥45 years. Notably, after 15 years of follow-up its cumulative incidence was 12 times higher than that observed in the group of patients whose onset was at age <10 years (Table 2 and Figure 1).

In the univariate analysis, male gender, smoking, SBP, and HDL-cholesterol at onset were significantly associated with the risk of DR throughout follow-up. However, the association of HbA1c with DR development was only marginally significant ($p = 0.079$). Remarkably, when taking the patients who were younger than 10 at onset as the reference group, the risk increased according to age at onset (Table 3). When an additional univariate analysis was performed for each variable according to its evolution along the follow-up period, a significant association with DR development was again observed for smoking, SBP, and HDL-cholesterol, with HRs

Table 3: Univariate analysis to analyze the association between age, gender, and other risk factors at T1D onset with the development of DR during the subsequent follow-up period.

Variable	HR (CI 95%)	p value
Sex		
Male	Reference	
Female	0.56 (0.39, 0.80)	0.001
Age (years)		
<10	Reference	
10–14	2.58 (1.24, 5.37)	
15–29	3.64 (1.87, 7.10)	<0.001
30–44	4.23 (2.04, 8.76)	
≥45	5.32 (2.21, 12.84)	
Smoking		
No/ex-smoker	Reference	
Smoker	1.68 (1.16, 2.44)	0.007
BMI	1.12 (0.94, 1.33)	0.274
SBP (per 10 mmHg)	1.15 (1.02, 1.31)	0.034
DBP (per 10 mmHg)	1.12 (0.94, 1.33)	0.203
HDL (per 10 mg/dL)	0.75 (0.64, 0.88)	<0.001
LDL (per 10 mg/dL)	1.01 (0.97, 1.06)	0.606
Triglycerides (per 10 mg/dL)	1.01 (1.00, 1.02)	0.174
HbA1c (per 1%)	1.09 (0.99, 1.19)	0.079

The HR given for quantitative variables refers to increments of 10 units for all covariables except for HbA1c, for which it is referred to increments of 1% points. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoproteins; HDL, high density lipoproteins; HbA1c, glycated hemoglobin.

Table 4: Univariate analysis to analyze the association between time-dependent variables along the follow-up and the development of DR during the same period.

Variable	HR (CI 95%)	p value
Smoking		
No/ex-smoker	Reference	
Smoker	1.75 (1.24, 2.47)	0.001
SBP (per 10 mmHg)	1.28 (1.14, 1.45)	<0.001
DBP (per 10 mmHg)	1.75 (1.44, 2.12)	0.001
HDL (per 10 mg/dL)	0.78 (0.69, 0.88)	<0.001
LDL (per 10 mg/dL)	1.06 (1.00, 1.13)	0.052
Triglycerides (per 10 mg/dL)	1.04 (1.02, 1.06)	<0.001
BMI	1.10 (1.05, 1.15)	<0.001
HbA1c (per 1%)	1.22 (1.08, 1.37)	0.001

SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoproteins; HDL, high density lipoproteins; BMI, body mass index; HbA1c, glycated hemoglobin.

(95% CI) similar to the former ones. Unlike what happened when the analysis was performed using values at onset, DBP, triglycerides, BMI, and HbA1c were now significantly associated with DR. Finally, the association between LDL-cholesterol and DR was slightly above the limit of statistical significance [1.06 (1.00, 1.13), HR (95% CI), $p = 0.052$] (Table 4).

Table 5: Multivariate analysis to analyze the association of the development of DR with age at T1D onset and with DBP, HDL, and HbA1c along the follow-up.

Variable	HR (CI 95%)	p value
Age at onset (years)		
<10	Reference	
10–14	2.57 (1.15, 5.76)	
15–29	3.04 (1.44, 6.47)	0.012
30–44	3.35 (1.49, 7.56)	
≥45	3.78 (1.37, 10.41)	
DBP (per 10 mmHg)	1.55 (1.26, 1.91)	<0.001
HDL (per 10 mg/dL)	0.77 (0.68, 0.88)	<0.001
HbA1c		
≤7%	Reference	
7-8%	1.34 (0.72, 2.46)	
8-9%	1.69 (0.92, 3.11)	0.009
>9%	2.56 (1.38, 4.75)	

DBP, diastolic blood pressure; HDL, high density lipoproteins; HbA1c, glycated hemoglobin.

The multivariate analysis confirmed the trend observed in the univariate one and the risk of developing DR increased according to age at onset, albeit less markedly (Table 5). In any case, when focusing on pediatric age, children with onset at the peripubertal period exhibited a significantly higher risk when compared with those <10, the HR increasing more than 2-fold. In adult patients, the risk of DR development increased more than three times with respect to children <10, and, notably, it was almost 4 times higher in the group of patients ≥45 at onset [HR 3.78 (95% CI: 1.37–10.41)]. The multivariate approach confirmed that, among the time-dependent variables, DR development along follow-up was also significantly influenced by DBP, HDL, and HbA1c, the latter when values were above 9% (Table 5).

4. Discussion

The rate of DR increases with the age of diagnosis. Taking advantage of the follow-up of the patients included in the T1D Registry of Navarra, we have confirmed the influence of some controversial risk factors on the occurrence of DR. Remarkably, we describe for the first time that the risk of DR 15 years after diagnosis increases with increasing age of onset, being highest in those with onset at age ≥45.

HbA1c is the factor exhibiting the highest impact on the development of DR [3]. In the Wisconsin study [17], the HR (95% CI) to develop DR for patients with HbA1c from 9.5 to 10.5, compared with patients with HbA1c levels <9.5%, is 1.72 (1.34, 2.21). However, it rises to 2.41 (1.91, 3.06) when HbA1c ranges from 10.6 to 12% and is even higher, 3.65 (2.87, 4.65), for HbA1c values >12%. We cannot compare these results with ours, because our highest quartile of HbA1c is lower than the lowest of theirs, but both studies are consistent in showing the relationship between poor metabolic control, as measured by glycated hemoglobin, and DR development and progression. This relationship has been shown in several

publications based on national or regional T1D records, some of them recently [1, 18].

T1D duration is the other factor clearly related to the onset and progression of DR [3, 17], and in our series this association was also observed.

Regarding the factors most frequently discussed, our results match those published on the risk of DR [1, 3, 17], which is higher in men than in women, although this difference disappears when the effect of confounding variables is prevented by the multivariate analysis, suggesting that the risk is due to factors other than gender.

The relationship between BP and DR has been generally accepted [1], but results have not always coincided. Published data are influenced by the type of analysis and are divergent if analysis of BP is performed at baseline or throughout follow-up, if BP is stratified by ranges or by 10 mmHg increments, or if the comparison is only between hypertensive and normotensive patients [3, 17]. Different results have also been reported depending on how DR is evaluated: as a whole, or taking NPDR and PDR cases separately, or even if the progression from one to the other is analyzed. Our results largely match those of the Wisconsin study [17], although the latter treats NPDR and PDR as different entities and we have analyzed them in a single group since there were only 14 patients with PDR in the T1D Registry of Navarre.

The association between smoking and DR is controversial: Hammes et al. [3] found a significant relationship, while other authors disagree [19]. Our results are in accordance with those of the Linkoping Diabetes Complications Study [20] in that although smoking is associated with the risk of DR in the univariate analysis, there is no relationship between both variables after adjusting for confounding factors in the multivariate analysis. Therefore, there must be other factors closely associated with smoking to explain this recurrent finding. The fact that smoking is linked with a worse glycemic control may be responsible for such an observation [21].

Dyslipidemia, especially increased triglyceride and decreased HDL-cholesterol levels and, to a lesser extent, the increase in total cholesterol and LDL-cholesterol, is a risk factor, albeit weak, for the occurrence of DR, especially in its severe forms [22, 23]. Thus, additional factors (genetic, inflammatory, and metabolic) might be necessary for lipids to induce such effect. In any case, there is experimental evidence that increased and biochemically altered plasma lipids lead to cytotoxicity in retinal capillary cells [3]. In our study the findings on LDL- and HDL-cholesterol and triglycerides (Tables 3, 4, and 5) are consistent with the data published on a Finnish population [22].

The occurrence of DR (PDR plus NDRP) has been related to high BMI in the DIS study in Sweden [1], but, in Finland, Hietala et al. [4] have not found any influence of BMI on the risk of PDR. Our results, in the multivariate analysis, differ from those obtained in Sweden, perhaps due to a smaller number of events in our series (135 versus 247 patients).

The influence of age of diagnosis on the onset and progression of DR has been the subject of numerous publications; most of them focused on children and the probable protective effect of the prepubertal period. Ours is the first study to include patients who have been diagnosed with T1D over the age of 40.

In our series, patients within the pubertal period, that is, children aged 10–14 years, exhibit a risk of developing DR which is 2.5 times higher than the risk associated with children who are aged 0–9 years. These data are consistent with the results of most authors [5–8], especially those of Olsen et al. [10], but differ from those obtained by Holl et al. [9] and by the Wisconsin study [24].

There is a controversy about the effect of hyperglycemia on the occurrence of microvascular complications before puberty. Discrepancies may be due to different interpretations about what is the exact range of age encompassed by the term puberty, which have led to heterogeneous ways of grouping patients aged <15 years. There is a real need to gain knowledge on this topic to take advantage of the age at diagnosis to help in deciding the extent of the aggressiveness of insulin treatment [2].

During puberty, glycemic control is worsened and the risk of microangiopathy development increases [25], which is partly due to insulin resistance. The insulin sensitivity in the middle of puberty is reduced by 30–35% compared to the late stage of puberty, prepubertal childhood, or adulthood. This seems to be mainly due to the effects of the growth hormone (GH) and the insulin growth factor-1 (IGF-1) [11]. The actions of GH are mediated by IGF-1, and the levels of both molecules have been correlated with the thickness of the capillary basement membrane as well as with diabetic angiopathy. The increased GH and IGF-1 during puberty are related with the increased gonadal steroids. Furthermore, the increased levels of sex hormones are directly related to vascular structural abnormalities associated with diabetes complications. This is due to the ability of such molecules to increase polyol metabolism in the basement membrane, as has been demonstrated in animal models [26]. There is an association between capillary basement membrane thickening and the duration of T1D in the postpubertal period. In fact, diabetes control markers, such as HbA1c and fasting glucose, are positively correlated with thickness in postpubertal but not in prepubertal patients, thus suggesting an interaction between diabetes control and puberty [8]. Psychosocial factors associated with adolescence may also contribute to a poorer glycemic control during this phase and thus to its subsequent undesirable effect on morbidity in patients with T1D [9].

Our patients aged 15 to 29 years have a risk of DR which is 3-fold the risk exhibited by children <10. Not surprisingly, these results are again in agreement with those obtained by Olsen et al. from the Danish Study Group of Diabetes in Childhood [10] and in disagreement with those recorded in the Wisconsin study [17]. In our series, the risk continues to increase with age, so that it is even higher for patients >30 and, especially, almost 4-fold, for patients ≥45. Hammes et al. [3] also found an increased risk for PDR in patients diagnosed with T1D and aged 15 to 40 years. By contrast, Kullberg et al. [12], studying patients aged up to 36 at onset and also gathering all forms of retinopathy, observed a decrease in the prevalence of DR in patients older than 30. In the Finnish records [4], the risk of PDR was lower in patients who were

aged 15 to 40 at onset compared to those whose onset was at puberty. The beta cells are deemed to be best preserved when diabetes onset is in adulthood. As a consequence, the glycemic control would be easier when development of T1D was at an age long after puberty, and this would explain the lower risk of DR found in these patients. Our group had previously published [27] that, in the patients included in the T1D Register of Navarra, a relationship between the age of onset and glycemic control also exists. However, in our cohort the worst glycemic control at follow-up was observed in the group of oldest patients at T1D onset. Thus, from this point of view our present findings about age at onset and risk of DR are consistent with our previous observations.

The current study is not exempt from limitations. The main one is that the number of patients who developed PDR was rather small in our cohort. For this reason, we were not able to analyze separately NPDR and PDR; that is, we do not have information about whether or not there are risk factors that influence differently the progression to one entity or to the other. We are confident that longer follow-up, lasting over 20 years, will allow us to obtain more information in the future.

We also consider that there are strengths in this work. Among them, we want to remark that our cohort includes a significant number of patients who were followed up from diagnosis. In fact, the onset of disease of each patient is the time of his/her inclusion in the study, which allows the analyses of incidence at different times, that is, 5, 10, or 15 years, to be more accurate than if the onset and baseline had not matched. Furthermore, we consider that the risk of bias that could have led to inaccurate results is rather low since the follow-up of all patients took place at the same hospital, and the screening and grading for the presence of DR were always performed by the same ophthalmologists, who followed similar clinical criteria throughout the study.

In sum, the analysis of the T1D Registry of Navarra shows, for the first time, that there is a relationship between the age at T1D diagnosis and the prevalence of DR throughout life and confirms the influence of T1D duration and glycemic control on the appearance of DR. Our results also support the view that BP and lipid profile have an effect on DR.

Competing Interests

The authors declare that there are no competing interests.

Acknowledgments

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Research Article

Heart Rate Variability as Early Biomarker for the Evaluation of Diabetes Mellitus Progress

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According to the American Diabetes Association (ADA), the side effects of diabetes mellitus have recently increased the global health expenditure each year. Of these, the early diagnostic can contribute to the decrease on renal, cardiovascular, and nervous systems complications. However, the diagnostic criteria, which are commonly used, do not suggest the diabetes progress in the patient. In this study, the streptozotocin model in mice (cDM) was used as early diagnostic criterion to reduce the side effects related to the illness. The results showed some clinical signs similarly to five-year diabetes progress without renal injury, neuropathies, and cardiac neuropathy autonomic in the cDM-model. On the other hand, the electrocardiogram was used to determine alterations in heart rate and heart rate variability (HRV), using the Poincaré plot to quantify the HRV decrease in the cDM-model. Additionally, the SD1/SD2 ratio and ventricular arrhythmias showed increase without side effects of diabetes. Therefore, the use of HRV as an early biomarker contributes to evaluating diabetes mellitus complications from the diagnostic.

1. Introduction

The diabetes mellitus (DM) is considered like an important economic and social problem owing to the long-term complications such as premature death [1, 2]. Furthermore, the DM complications like neuropathies and failure renal increase the morbidity and cardiovascular mortality [3]. For this reason, the global healthcare expenditure on complications rises each year [4].

The ADA recommended some medical and laboratory tests to diabetes diagnostic [5]; however, none of them is considered useful to evaluate the time course and damage caused by diabetes including renal and nervous systems injury and cardiovascular disease which are regarded as chronic diabetes mellitus (cDM) complications [2, 3, 6]. Therefore, it is necessary to determine if the DM is chronic or acute for the best diagnosis and prognosis of illness [7].

On the other hand, the study of the alterations on DM the murine family has been widely used because these animals are handy and susceptible to DM development. According to the literature, the DM models induced with a streptozotocin single dose (100–200 mg/kg i.p.) [8] showed mortality more than 20% in the first week after administration. Additionally, insulin administration over time is extremely necessary for animal's survival [9, 10].

The diabetic model showed different physiological alterations when insulin is administered [11]. For this reason, it is important to develop a DM model with longer time

period survival, lack of insulin, and a noninvasive tool to determine an early diagnostic. The heart rate variability (HRV) was proved to be a noninvasive tool as valuable clinical evidence for the prognosis of cardiovascular events and several disorders [12, 13] although some studies revealed a lower HVR, associated with sudden death in humans [14, 15].

One way of the HRV measurement is done by the RR Poincaré plot, quantifying long (SD2) and short (SD1) term and SD1/SD2 (iHRV) ratio. These parameters could help prognosis in a variety of pathological entities [15, 16].

The aim of this study was to identify an early biomarker in clinical practices for the evaluation of DM progress as an adequate and effective technique, using a cDM-model without insulin administration.

2. Methods

2.1. Animal Model. Adult male mice CD1 of 8 weeks old with 33 g of weight in average were used. All the animals were maintained with a 12 : 12 h light-dark cycle (7:00–19:00) and allowed free access to pellets LabDiet 5001 and water. All the experiments conducted on mice were approved by the Animal Care Committee of the Instituto de Fisiología Celular, Universidad Nacional Autónoma de México. Animal care was supported by the "International Guiding Principles for Biomedical Research Involving Animals," Council for International Organizations of Medical Sciences, 2013.

2.1.1. Induction of Diabetes Mellitus. One week before induction of diabetes, oral rehydration salts (NaCl 3.5 g/L, KCl 1.5 g/L, and $Na_3C_6H_5O_7$ + H_2O 2.9 g/L and glucose 20 g/L) were given to the mice, supplemented with 10% glucose to prevent fatal hypoglycemic [8].

The animals were kept in fasting for 4 hours before induction. The DM induction was a single injection of 120 mg/kg i.p. streptozotocin (STZ) (Sigma-Aldrich) [17]. The STZ was dissolved with 0.12 mL of isotonic saline solution to pH = 7.4 instead of sodium citrate; during the preparation, the room remained in the darkness. This maneuver did not last more than 15 minutes [8].

After administration, in the first week the mice consumed oral rehydration salts supplemented with 10% glucose. This allowed the lack of dehydration and hypoinsulinemic and hypovolemic shock in the process [6, 10]. In addition to the survival, oral rehydration salts were provided to the mice during the following nine weeks.

2.1.2. Blood Collection and Clinical Chemistry. The mice were euthanized, 10 weeks after administration, due to the complications on electrical activity of heart, but not necessarily the vascular system [18]. The blood samples were obtained 1 minute before the euthanasia between 8:00 and 10:00 a.m., not maintaining animals fasting for metabolic parameters measurement. The glucose plasma, cholesterol high density lipoprotein cholesterol (HDL-c), and cholesterol quantification were determined by glucose oxidase, phosphotungstate, and cholesterol oxidase technical, respectively; lipoprotein lipase assays were used for triglycerides and chemiluminescence for insulin.

2.1.3. The Metabolic Cage. After 9 weeks of administration, mice were placed in the metabolic cage for 24 hours (12 hours of light and 12 hours of darkness) to quantify urine, feces, water, and food intake. The visual exam and dipstick test were used for urinalysis (Combi sys plus screen 11; Analyticon). Previously, the mice were handled and placed in the metabolic cage for two hours daily during 5 days prior to test to avoid stress.

2.2. Chronic Diabetes Mellitus Evaluation: Electrocardiogram Record. Ten weeks after induction, the mice were anesthetized with pentobarbital sodium 0.63 g/kg i.p. and placed in supine position for recording ECG for 30 minutes. The bipolar ECGs were recorded with subcutaneous needle electrodes in the configuration lead I. The electrodes were placed in right and left in the fourth intercostal space. The ECG signal was amplified 700 times and filtered at 60 Hz. The signal was recorded on personal computer at sampling frequency 1 KHz and analyzed offline with Clamp Fit® program (Molecular Device).

The analysis of heart rate variability (HRV) was made with RR interval (RR_i) time series. The ECG was recorded for thirty minutes and 100 values of RR_i were randomly chosen. The RR intervals were measured between consecutive beats [19]. Also, the QTc was calculated with the corrected Bazett formula [20]. All mice were continuously monitored to guarantee adequate ventilation and temperature.

2.3. Heart Rate Variability. To quantify the HRV time domain, the heart rate, SD1, SD2, and iHRV were calculated. The RR_i interval is the time between the maximum value of the QRS_i complex and the next maximum value of the QRS_{i+1} complex. The Poincaré plotted the RR_{i+1} interval as function of the previous RR_i interval. The heart rate is the inverse RR interval. SD1 is the standard deviation of the distances between all points of the Poincaré diagram and $RR_{i+1} = RR_i$ line. SD2 is the standard deviation of the distance between all points of the Poincaré diagram and $RR_{i+1} = -RR_i + 2\overline{RR}_i$ line where $\overline{RR}_i$ is the average value of all RR_i [16]. iHRV is the SD1/SD2 ratio which is the value, thus suggesting the delicate equilibrium between sympathetic and parasympathetic systems on heart [14, 21–23].

2.4. Data Analysis and Statistics. All data is presented as the mean ± standard error. The t-test was used for data analysis; the values were considered statistically significant if value was lower than 0.05 denoted by $*$. The analysis was done with program Origin Pro version 8.0 Lab Corporation.

The distances for obtained SD1 and SD2 were calculated with next equations:

$$\sqrt{\left(\frac{RR_i - RR_{i+1}}{2}\right)^2}, \quad (1)$$

$$\sqrt{2\left(\frac{2\overline{RR}_i - RR_i - RR_{i+1}}{2}\right)^2}. \quad (2)$$

With all distances (1) and (2) equations, the SD1 and SD2 standard deviations were determined, respectively.

3. Results

3.1. Characterization of Diabetes Mellitus Chronic Model. In this research, 22 mice were used for cDM-model and 20 for control. All the injected animals had glycosuria, ensuing seven days after induction with mortality of 10%. Also, the control animals showed a growth on weight from 35 ± 0.5 g to 39.5 ± 0.7 g in the following 10 weeks after the injection while the cDM-model presented lowering weight at 33.6 ± 0.7 g in the last week. The loss of weight was evident from third week after injection of STZ (Figure 1(b)). In both conditions, after the tenth week of injection, the plasma glucose was measured; all the obtained values showed two normal distributions and were characterized by a mean of 161.6 ± 46.8 mg/dL and 730 ± 123.2 mg/dL (fitting results). The first normal distribution corresponds to the glucose of the control, and the second normal distribution is the glucose of injected animals. Consequently, the concentration of control glucose is considered if the values were from 68.1 to 255.1 mg/dL whereas the values of glucose in the diabetic were from 483.6 to 976.4 mg/mL (cDM-model). If the value of glucose was more than the control but less than the diabetic, the mouse was regarded hyperglycemic, not yet diabetic (Figure 1(a)).

The cDM-model had plasma glucose of 769 ± 216 mg/dL; hence the animals were diabetic. These had an insulin decrease of 7-fold, considering dyslipidemia like in humans. The cholesterol had 81.7 mg/dL and triglycerides 76 ± 8 mg/dL in the control, and in cDM-model 163 ± 19 mg/dL cholesterol and 118 ± 17 mg/dL triglycerides were presented. The HDL-c lipoprotein decreased by 39% in cDM-model and the LDL values was obtained by Friedewald formula because it is theoretical [24]. This value in control was 2.4 mg/dL and 100 mg/dL in cDM-model (Figure 1).

3.2. Metabolic Cage. In cDM-model, the volume of water intake had a 10-fold increase as excreted urine volume; the food intake grew by 60% and defecated by 107% more than control (Table 1). The urinalysis showed that both groups had clear urine. Furthermore, the dipstick tests showed glycosuria and increase in the blood, ketones, proteins, nitrites, and leukocytes in the cDM-model (Table 1).

3.3. Evaluation Progress DM: Electrocardiogram. The HR was not affected by the diabetes mellitus because the HR in control mice had 482 ± 5 bpm and cDM-model had 488 ± 5 bpm ($p < 0.05$). However, 42% of diabetic mice exhibited supraventricular arrhythmias (8% of inversion in P-wave and 33% of P-notched). The 67% of diabetic mice showed ventricular arrhythmias like 50% QRS-complex inversion and 33% T-wave height of the animals. Also, 8% decrease of both T-wave and QRS-interval amplitude was shown, presenting 8% block of second-degree arrhythmia (Figure 2).

Table 1: Characterization of diabetes in cDM-model.

Metabolic parameters		
	CT ($n = 33$)	cDM-model ($n = 21$)
Water intake (mL)	6 ± 0.4	60 ± 7*
Food intake (g)	5 ± 0.2	8 ± 0.7*
Feces excreted (g)	1.8 ± 0.2	5 ± 0.5*
Urine excreted (mL)	0.4 ± 0.1	40 ± 6*
Clinical biochemistry values		
Serum electrolytes	CT ($n = 5$)	cDM-model ($n = 6$)
Sodium (mmol/L)	149 ± 1	154 ± 4
Potassium (mmol/L)	9 ± 0.5	11 ± 0.4
Chloride (mmol/L)	115 ± 2	115 ± 4
Calcium (mg/dL)	9.4 ± 0.2	9.5 ± 0.6
Urine test strip		
	CT ($n = 21$)	cDM-model ($n = 20$)
Ketones	Negative (100%)	0.1 ± 0.06 (20%)
Glucose (mg/dL)	Negative (100%)	722.5 ± 149.7 (100%)
Protein (mg/dL)	Traces (47%)	24 ± 6 (85%)
Blood (ery/μL)	Negative (100%)	16.1 ± 4.54 (50%)
pH	6.40 ± 0.14 (100%)	6.05 ± 0.2 (100%)
Nitrites	Positive (9%)	Positive (43%)
Leukocytes (leuk/μL)	Negative (100%)	30.83 ± 8 (30%)
Specific gravity	1.02 ± 0.001 (100%)	1.01 ± 0.0006 (100%)
Urobilinogen	Negative (100%)	Negative (100%)
Bilirubin	Negative (100%)	Negative (100%)

The data obtained on metabolic cage and urine of 24 hours. The values are described as mean ± SEM. *Student's t-test $p < 0.05$.

Other studies showed sympathetic and parasympathetic modulation on heart rate, the influence of nervous system on frequency and variation (SD1, SD2 and SD1/SD2 ratio) was altered from one disease to another, and an aging process [25, 26]. In this case, a Poincaré plot was used to establish the HRV for diabetic chronic. The alterations presented, matching in 3–5 stages of chronic disease which are associated with accelerated cardiovascular diseases like coronary disease [27].

The diabetic mouse had a decrease in the HRV (Figure 3). In control, SD1 was 1 and SD2 was 1.3; in diabetics SD1 was 0.9 and SD2 was 0.8. The iHRV was increased from 0.8 to 1.1 in cDM-model (Table 2). The heart rate of the control before and after administration was 275 ± 86 bpm with iHRV of 0.8.

The SD1 and SD2 parameters before the STZ-injection (time 0) were SD1 = 14 and SD2 = 18; after ten weeks they reduced to SD1 = 1 and SD2 = 1.3; however, the iHRV value remained constant (0.8), implying the nonexistence of changes in the delicate balance between sympathetic and parasympathetic systems by aging [23, 28].

Other reports associate the QTc prolongation and vascular diseases with risk factors of 92% increase in mortality and decrease the survival in the forthcoming 8 years in diabetic patients [29]. In cDM, the RR interval did not change; however, the QT interval increased by 11%, comparing with control. In addition, the QTc was prolonged to 17% (Table 2) without vascular troubles because the ratio heart mass/body

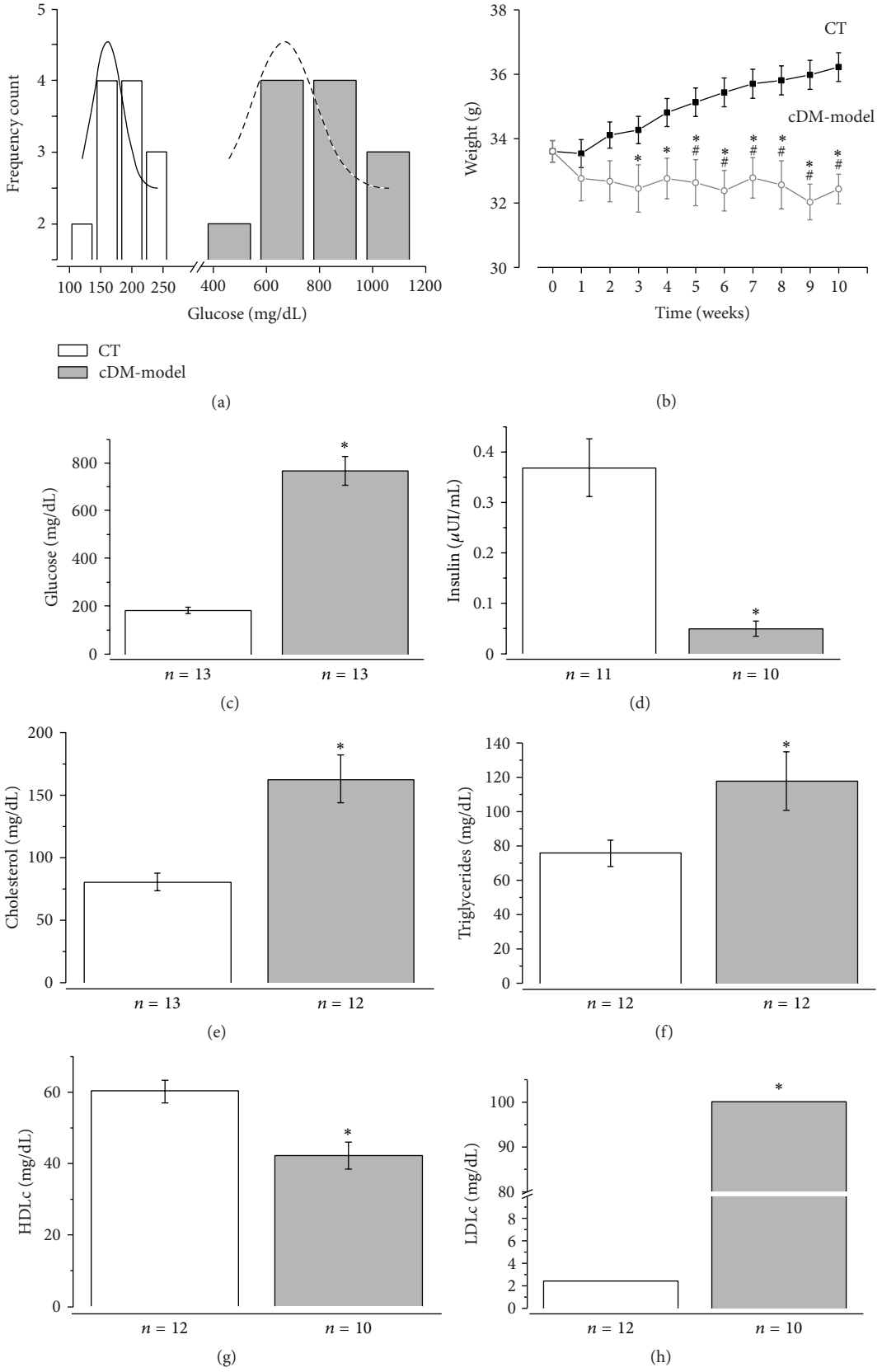

Figure 1: Characterization of chronic diabetes mellitus model. (a) Distributions of glucose plasma. (b) The plot shows that the cDM-model mice are losing weight since the third week after STZ-injection. (c) Increased plasma glucose. (d) Decreased insulin plasma. (e–h) Nonfasting lipid profile (LDL, HDL, total cholesterol, and triglycerides) is altered for the treatment. n = animals number, $^{*}p$ (<0.05) versus control (dark line), and #: versus first weight (grey line).

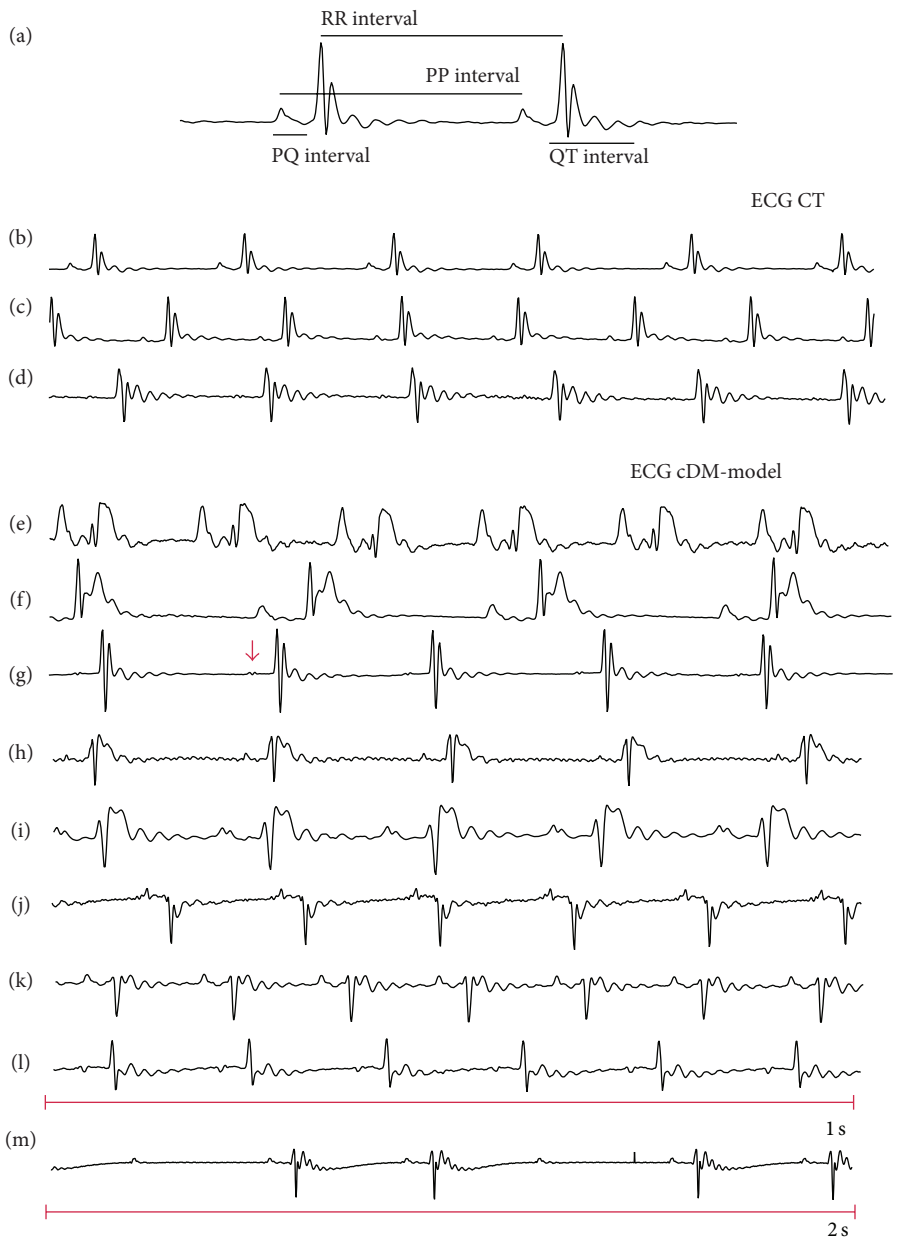

Figure 2: Electrocardiogram recording. (a) ECG intervals (b, c, d) show ECG control conditions. (e–m) ECG from cDM-model. (e, g, h) and (i) have an elevated ST segment. (j) and (k) have a depression QRS complex. (l) P-wave inverted. (g) The arrow show p-notched. (m) Second-degree block. The segments have 1 second of record except in (l) have 2 seconds because block produced intense bradycardia.

weight was similar (control $n = 18$, $80 \pm 2.6 \times 10^{-4}$; cDM $n = 15$, $84 \pm 4.3 \times 10^{-4}$).

4. Discussion

In the literature, the STZ-models are considered diabetic when the plasma glucose was greater than 270 mg/dL [8, 10]; using the suggestions of the ADA to evaluate the diabetic animals, the mice showed dyslipidemias and impaired plasma glucose [30]. Additionally, our model presented erythrocytes and leucocytes with the absence of the bilirubin and urobilinogen (Table 1) in the urine test. The results demonstrated chronic diabetes in the cDM with an infection in the urinary tract without injury on the vascular, renal, and hepatic systems. These signs presented some similarities to chronic diabetes patient in second stage, developed in kidney disease due to the fact that these animals had proteins and blood in urine. The DM patients, who showed proteins and blood in urine, should have approximately from five to ten years of diabetes [31]. These conditions supported chronic DM, and these individuals usually showed kidney failure, in the ensuing 15 to 25 years [32].

In this proposal, the cDM-model has polyuria, polydipsia, and polyphagia, clinical signs which are exhibited in the

Table 2: Heart rate variability.

Interval (ms)	SD1	SD2	Poincaré index	
			SD1/SD2	Variability
RR				
Control = 126 ± 1.6	1	1.3	0.8	SD1 10%; SD2 39%
cDM-model = 125 ± 1.2	0.9*	0.8*	1.1∞	SD1/SD2 38%
QT				
Control = 39.3 ± 1.1	2	3.5	0.6	SD1 60%
cDM-model = 45.3 ± 0.8∞	0.8*	3.4	0.2*	SD1/SD2 60%
QTc				
Control = 35 ± 1.6	0.03	0.2	0.15	SD2 300%
cDM-model = 41 ± 0.7∞	0.03	0.6∞	0.05*	SD1/SD2 33%

Control, $n = 10$; cDM-model, $n = 21$. Control * decrease, ∞ increase; Student's t-test $p < 0.05$.

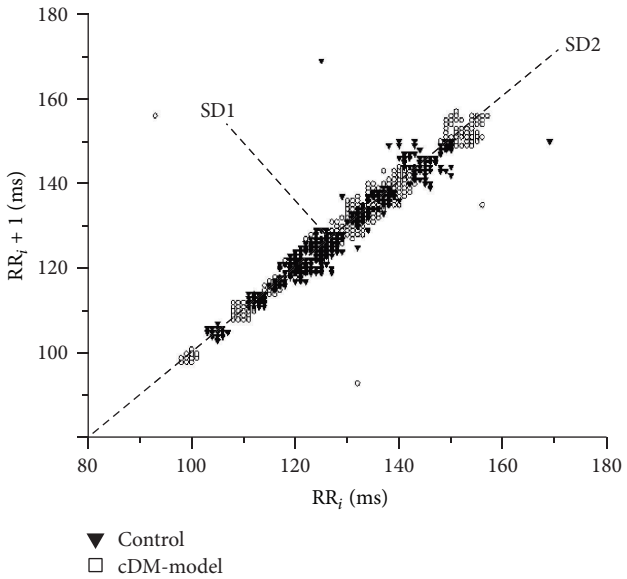

Figure 3: The Poincaré plots. The plot was constructed with RR interval showing an increase in SD2 in diabetic animals. The open square shows values from cDM-model and control in dark triangle.

chronic diabetic patients [30]. The diabetic animals did not present arrhythmias by dehydration in plasma because the electrolytes did not change in the cDM (Table 1).

Previous studies have shown that the dynamic HRV is a cut-off point to imbalance the nervous system on renal, cardiovascular, and endocrinology systems [22]. The R-R fluctuations allowed identifying some alterations in those systems. However, recent investigations showed that the HRV data are associated with the healthy subjects or chronic disease with side effects. Thus, in our model, the SD1 and SD2 alterations were determined without side effects (see above).

Additionally, the cDM-model showed variability in the dynamic RR interval which was associated with supra- and ventricular arrhythmias (Figure 3). In Poincaré plot of the QT segment plot, an increase was observed in SD2, related to lethal ventricular arrhythmias. Loss on the activity of autonomic system is shown. The long-term variability or SD2 was lower in 48% and the Poincaré (SD1/SD2) index was 38% higher than control. Our study implied that the animals could be in early stages of the nephropathy [33, 34], besides ventricular arrhythmias.

5. Conclusion

In summary, in our cDM-model, ventricular arrhythmias were shown, associated with long-term QTc, causing the increase of comorbidity and sudden death [35]. These arrhythmias are correlated to the potassium currents alterations in mice with the same treatment [11]. The changes on the total current of the membrane were associated likewise with alterations in the iHRV [36].

Further, the heart rate did not change in cDM, suggesting that diabetes, in this step, did not present even cardiac autonomic neuropathy [37] because of the iHRV remaining with the influence of sympathetic and parasympathetic tones [38]. This research demonstrated that the first damage was caused on cardiac electrophysiology by diabetes, before neuropathies and nephropathies. The electrophysiological changes in the cDM-model were demonstrated to be independent of vascular, infectious processes and disturbances in electrolytes. The current analysis proposed that SD1 and SD2 and SD1/SD2 ratio are early biomarkers for evaluating the progress of diabetes.

Additional Points

Clinical implications of this study are as follows. Consistent with our recent study findings, a noninvasive, straightforward, and inexpensive method was developed. On the other hand, this data treatment only requires arithmetic calculus, being a better procedure of DM chronic diagnostic and health care, preventing side effects in DM patients.

Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interests.

Authors' Contributions

Rosa Elena Arroyo-Carmona designed these experiments; Rosa Elena Arroyo-Carmona, Ana Laura López-Serrano, and David Medel-Cajica took responsibility in collecting data. The data analysis was done by Rosa Elena Arroyo-Carmona, Alondra albarado Ibañez, Francisca María Fabiola Mendoza-Lucero, Ruth Mery López Moyorga, and Julián Torres Jacome. All authors contributed to drafting or revising the paper and all authors approved the final version of the paper.

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Research Article

Epigenetic Studies Point to DNA Replication/Repair Genes as a Basis for the Heritable Nature of Long Term Complications in Diabetes

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Metabolic memory (MM) is defined as the persistence of diabetic (DM) complications even after glycemic control is pharmacologically achieved. Using a zebrafish diabetic model that induces a MM state, we previously reported that, in this model, tissue dysfunction was of a heritable nature based on cell proliferation studies in limb tissue and this correlated with epigenetic DNA methylation changes that paralleled alterations in gene expression. In the current study, control, DM, and MM excised fin tissues were further analyzed by MeDIP sequencing and microarray techniques. Bioinformatics analysis of the data found that genes of the *DNA replication/DNA metabolism process* group (with upregulation of the *apex1, mcm2, mcm4, orc3*, *lig1*, and *dnmt*1 genes) were altered in the DM state and these molecular changes continued into MM. Interestingly, DNA methylation changes could be found as far as 6–13 kb upstream of the transcription start site for these genes suggesting potential higher levels of epigenetic control. In conclusion, DNA methylation changes in members of the *DNA replication/repair process* group best explain the heritable nature of cell proliferation impairment found in the zebrafish DM/MM model. These results are consistent with human diabetic epigenetic studies and provide one explanation for the persistence of long term tissue complications as seen in diabetes.

1. Background

Hyperglycemia in patients with diabetes mellitus (DM) (both types 1 and 2) leads to a multitude of complications such as cardiovascular disease, aberrant angiogenesis, retinopathy, nephropathy, neuropathy, and impaired wound healing [1]. Our laboratory has previously reported an adult zebrafish model of type 1 DM that can be used to study the mechanisms underlying the long term complications of the disease. In this model, streptozocin (STZ) induced hyperglycemia (serum glucose = 315 ± 40.96 mg/dL) is accompanied by the full range of diabetic complications seen in patients with DM [2, 3]. Additionally, we have shown that withdrawal of STZ results in regeneration of pancreatic β-cells and the return of previously diabetic fish to a physiologically normal glycemic state within 2 weeks (serum glucose = 62.5 ± 13.6 mg/dL); however, in contrast, the tissue deficits associated with hyperglycemia persisted permanently (e.g., impairment of angiogenesis [4], impairment of skin wound healing [3], and impairment of limb regeneration [3]). Of relevance to this study, fin regeneration in the DM and MM states has been shown to be significantly impaired and that impairment was found to be due to a decrease in cell proliferation of the fin tissue [2, 3]. Multiple controls indicated that this was a direct effect of β-cell necrosis-induced hyperglycemia and not due to any secondary effects of STZ treatment [2, 3]. These findings are consistent with large scale DM clinical trials whose results indicate that

once initiated, diabetic complications persist and continue to progress unimpeded even when glycemic control is achieved through pharmaceutical intervention [5–8]. Additionally, this persistence in diabetic tissue deficits has also been supported by multiple lines of experimental laboratory evidence [3, 9–13] and collectively, these data indicate that the initial hyperglycemic period results in permanent abnormalities in the target organs. This harmful phenomenon has been termed metabolic memory (MM) [14, 15] and to date, the underlying molecular mechanism(s) of MM remain unknown.

In the current study, we focused on the molecular mechanisms underlying DM and MM as studied in limb tissue (caudal fin) using our DM/MM zebrafish model because the fin is impaired in its ability to regenerate in the DM and MM states and this impairment was due to a decrease in cell division rates. The objective of the current study was to determine (1) what functional gene groups are prominent during DM that best explain the heritable nature of tissue deficits observed in this model, (2) which genes of these groups persist into MM, and (3) where DNA methylation changes occur in these genes. We found that the zebrafish DM/MM states are associated with changes of the *DNA replication/DNA metabolism process* group. These changes involved (1) upregulation of specific genes of this group (*apex1, dnmt1, mcm2, mcm4, orc3*, *pola2*, and *lig1*) in the DM and MM states and (2) alterations in the DNA methylation patterns of all of these genes. Differentially methylated DNA regions (MRs) were found as far as 6–13 kb upstream of the transcription start site for the affected genes but were also found within the gene proper in one case.

The overriding hypothesis of these studies was that "*persistent tissue changes in the DM/MM states correlate with hyperglycemia-induced DNA methylation changes that are associated with specific functional gene groups related to the heritable nature of MM*." To test this hypothesis we employed (1) MeDIP sequence analysis in combination with concomitant gene expression analysis, (2) *in silico* determination of prominent functional groups of differentially expressed genes observed in the DM and MM states, and (3) further bioinformatics analysis of methylated genomic regions upstream and downstream of the transcription start site of those genes that had been identified. This approach has allowed us to gain insight into the underlying epigenetic mechanisms that might explain the persistence of impaired tissue function(s) that occur in the DM state and continue into the MM state using the zebrafish diabetic model. The study focused on the zebrafish caudal fin because previous studies have established that fin tissue is best suited for experimental creation of a "pure" metabolic memory tissue [2, 3]. Other tissues of the zebrafish (e.g., kidney, retina, and skin) enter the MM state following β-cell regeneration, but unlike the fin; with these it is more difficult to form a tissue that lacks the residual molecules that were created in the original hyperglycemic state such as ROS (Reactive Oxygen Species) and AGEs (Advanced Glycation Endproducts). The presence of such pathology-inducing molecules as ROS and AGEs introduces complicating variables that makes evaluation of the epigenetic effects more difficult to interpret.

2. Methods

2.1. Zebrafish Husbandry, STZ Injection, and Fasting Blood Glucose Determination. The maintenance of zebrafish stocks (*Danio rerio*), the induction of hyperglycemia, blood glucose determinations, and fin regeneration methodology were performed as previously described [2]. For intraperitoneal injection an insulin syringe with a 28.5-gauge needle was used to deliver 0.3% streptozocin (STZ) (Sigma, S0130) solution in 5 mM citrate buffer, pH 5.0 to a dose of 350 mg/kg (70–150 microliters dependent on weight). Control fish were injected with a like volume of citrate buffer. The fish used in these studies were approximately 4–7 months of age. Fasting blood glucose level parameters used included the following: normal, 60 mg/dL; DM, 315 mg/dL; and MM, a return to 60 mg/dL. All procedures were performed following the guidelines described in "Principles of Laboratory Animal Care" (NIH publication number 85-23, revised 1985) and approved IACUC animal protocols 08-19 and B11-16 for these studies.

2.2. Creation of a "Pure" Metabolic Memory Tissue for Molecular Analysis. As described in previous studies [2, 3], we have designed a way to obtain MM tissue that lacks any residual molecules that are created in the original DM state such as ROS and AGEs (common to diabetes in all vertebrate species [16]). As shown in Figure 1, this involves repeated amputation and regeneration of fin tissue that has entered the MM state following regeneration of β-cells. As stated, analysis of this MM fin tissue finds that it lacks any of the residual molecules found generated in the original DM state so that this regenerated fin tissue is in a "pure" MM state without the complicating signals existing in the original hyperglycemic DM tissue [2, 3].

2.3. RNA Extraction. RNA extraction procedures followed that reported previously by our laboratory without exception [2, 3, 17]. Triplicate samples of 15 caudal fin samples were obtained from intact fin tissue for (1) acute diabetic fish (DM), (2) 60-day metabolic memory fish (MM), and (3) controls (CTRL). For clarification purposes, (1) CTRL fish represents normal adult zebrafish vehicle injected for the diabetic group, (2) DM represents zebrafish that have been induced into an acute diabetic state (for a three-week period) using STZ, and (4) MM represents zebrafish that were initiated and were maintained in a diabetic state for the three-week period but returned to a euglycemic state following withdrawal of STZ (referred to as MM fish throughout the paper). At 30 days after drug removal, the caudal fins were amputated to eliminate any residual molecules within the fin tissue from the original hyperglycemic period as previously described [2, 3]. After an additional 30-day growth phase, the fish were considered to be the final MM group to be used for various analyses of the excised "pure MM" fin tissue (see Figure 1).

2.4. Microarray Analysis. Extracted RNA (procedure described above) was used to probe the previously established

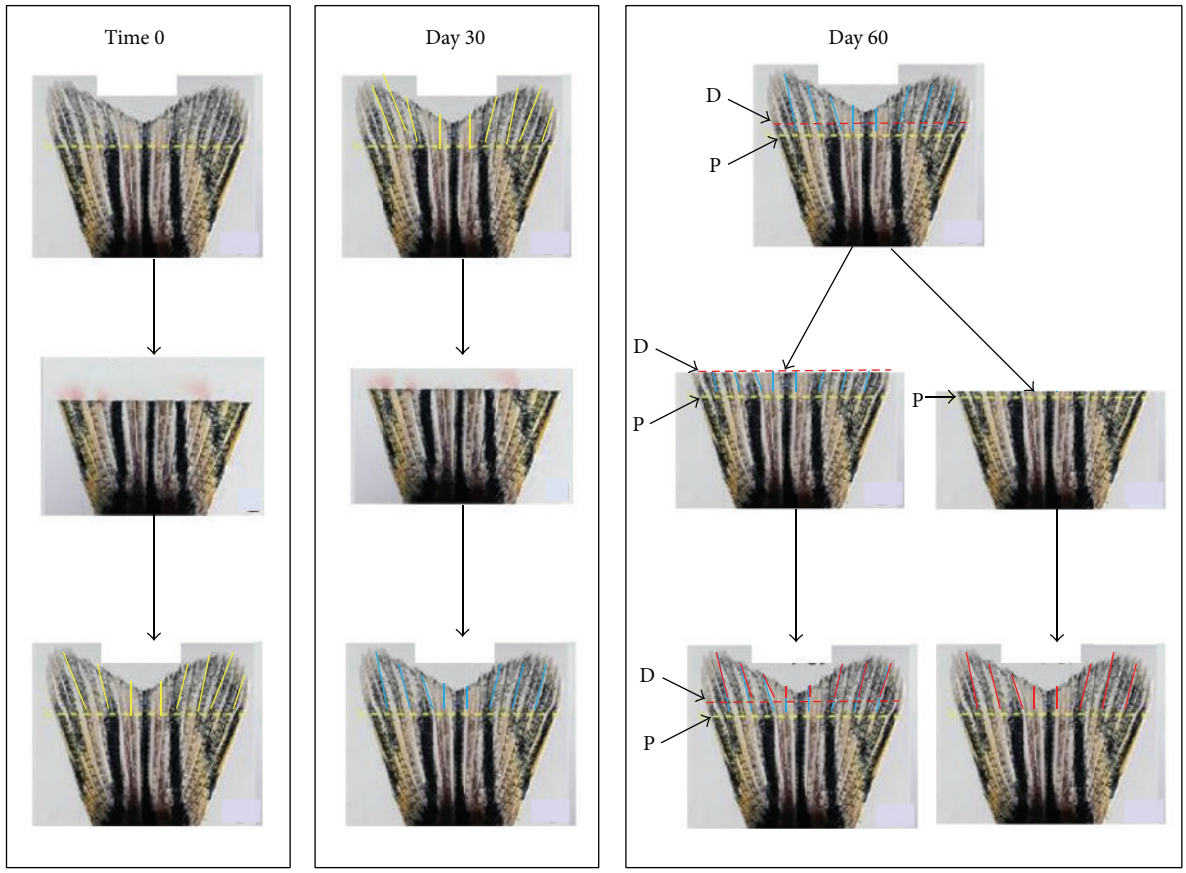

Figure 1: Schematic of amputation scheme employed to generate metabolic memory fin tissue. At Time 0, caudal fins were amputated and allowed 30 days for regrowth (Time 0 panel). At 30 days, the fins were again amputated and allowed an additional 30-day period of regenerative growth (Day 30 panel). This tissue is called metabolic memory tissue as it was generated outside of the hyperglycemic state. At 60 days, the groups were split into two fin sets and were cut either proximally (yellow dashed line, P) or distally (red dashed line, D) [Day 60 panel]. When analyzed for residual ROS and AGE molecules, the growth from the proximal and distal cuts (bottom left side of Day 60 panel, indicated by blue and red bars) had no residual ROS or AGE molecules, while tissue of the original fin (inferior to the proximal cut) did have residual ROS and AGE molecules [2, 3]. Excision of the distal cut growth (red bar area) was used for all DNA methylation sequence analysis and gene expression (microarray) analysis because it was pure metabolic memory region tissue (with no residual molecules from the original hyperglycemic state) and was clearly morphologically distinct from the original hyperglycemic fin tissue (each cut line can be observed in the regenerate fin tissue).

Affymetrix GeneChip® Zebrafish Genome Array which contains 15,509 probe sets designed to interrogate expression of 14,900 *Danio rerio* transcripts [2, 3, 17]. Microarray analysis was conducted according to manufacturer's instructions for the Affymetrix 3′ IVT Express Kit and all subsequent procedures were followed as previously reported by our laboratory [2, 3, 17] with the modification that (1) we first determined all genes with altered expression in the DM state (as compared to controls) and then from this group (2) we determined which of these genes maintained altered expression in the MM state. In our previous studies, this analysis was performed simultaneously for the DM and MM states [2, 3, 17]. This new method was utilized because it was more inclusive of all genes initially affected by hyperglycemia.

2.5. DNA Isolation and Methylated gDNA Sequencing. Triplicate samples of 15 caudal fins were obtained from control, DM, and MM zebrafish caudal fin tissue (conditions for control, DM, and MM were the same as described for Section 2.3) and immediately processed via the PureLink Genomic DNA Mini-Kit (Life Technologies). Methylation DNA immunoprecipitation sequencing (MeDIP) and initial sequence analysis was performed as previously described by our laboratory [2, 3, 17].

2.6. Gene Enrichment Analysis from Zebrafish Microarray Analysis. Gene function enrichment analysis was performed using DAVID Bioinformatics Resources 6.7 [18]. Additionally, the STRING 9.1 online bioinformatics resource was also utilized to visualize the results of this analysis, namely, representing relationships between specific genes and the significance of their interaction as described by Franceschini et al. [19].

2.7. Methylation Analysis of Zebrafish gDNA from Control, DM, and MM Groups. Analysis of FASTQ files generated by Illumina Genome Analyzer IIx was performed using Galaxy (https://usegalaxy.org/) as published by Goecks et al. [20]

and Blankenberg et al. [21]. The MACS algorithm [22] of the Galaxy program was specifically applied for analysis of DNA methylation changes among the groups studied.

The conditions for application of the algorithm were set with the following parameters: effective genome size: 1,480,000,000 bp; tag size: 25 or 32 depending on the results of quality control and trimming; band width: 300; P value cut-off for peak detection: $1e-05$; MFOLD: 30; regions around the peak region to calculate maximum lambdas local lambda: 1000, 5000, and 10000; mapping of methylated regions to zebrafish genome and visualization of the results were performed using UCSC genome browser and IGV genome browser as described by Thorvaldsdóttir et al. [23] and Robinson et al. [24].

3. Results

3.1. Genes of the DNA Replication and DNA Metabolism Functional Categories That Are Differentially Expressed in the DM State as Compared to Controls. Gene enrichment analysis was performed to determine the functional categories that were enriched by the genes that were identified in the microarray studies. Using a cut-off of 2-fold differential expression in the DM condition relative to control with a false discovery rate (FDR) cut-off of 0.05, we identified a number of functional groups (see Table 1). The first four groups were related to (1) *protein folding and binding*, (2) *RNA translation*, (3) *protein transport and localization*, and (4) *cell homeostasis*, while the next functional category was related to (5) the *DNA replication/DNA metabolism process* group. We focused further analysis on the latter group (enrichment score of 2.7, Table 1) because of its important relationship to understanding the mechanisms underlying the heritable nature of MM in the zebrafish. While the other groups would have a role in MM, they cannot explain the heritable basis of the cell proliferation deficits in MM.

We identified 51 genes as being overrepresented in the functional category *DNA replication/DNA metabolism process* group (Supplemental Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/2860780). Again, a parameter of at least 2-fold differential expression in the DM condition relative to controls with a FDR cut-off of 0.05 was used for gene selection.

We next determined that genes overrepresented in the functional category *DNA replication/DNA metabolism process* group do, in fact, have associations with each other based on analysis using the STRING 9.1 program that determines gene associations based on published human and mouse research literature [19]. The diagram shown in Figure 2 was generated by the STRING 9.1 program and depicts the interrelationship of all genes listed in Supplemental Table 1. It is to be noted that, among the 51 genes reported to interact in this group, only a subset were found to be upregulated in both the DM and MM states of our study as depicted by the diamonds in Figure 2 (discussed in more detail in Section 3.2).

3.2. Identification of Genes Differentially Expressed in the DM State That Remained Differentially Expressed in the MM State. Of the functionally related genes of Figure 2, we selected those genes that had altered expression in the DM state and then maintained this altered expression in the MM state (relative to controls) using a cut-off of 1.5-fold differential expression in the MM state condition with a FDR cut-off 0.05. A 1.5-fold differential expression cut-off was used for the genes affected in the MM state because it was consistent with the criteria applied to the analysis of the MM state in our previous published studies [2, 3, 17]. Additionally, this approach was chosen with the logic that MM would encompass genes whose altered expression in the DM state would abnormally persist in the MM state [2, 3, 17]. Six genes met this cut-off and are depicted in the heat map shown in Figure 3. These genes included *apex1*, *dnmt1, mcm2, mcm4, orc3*, *pola2*, and *lig1*. All of these genes were found to be upregulated. These genes can further be classified into two functional groups to include (1) DNA replication and/or repair (*apex1, mcm2, mcm4, orc3*, *pola2*, and *lig1*) and (2) DNA methylation (*dnmt1*, a methyltransferase that functions during the replication process) based on the criteria of the applied algorithms.

Table 1: Gene Ontology biological process categories enriched by genes differentially expressed in DM relative to controls.

Gene Ontology ID	Biological process
Enrichment score: 8.38	
GO:0051082	Unfolded protein binding
GO:0006457	Protein folding
Enrichment score: 4.08	
GO:0006412	Translation
Enrichment score: 3.95	
GO:0015031	Protein transport
GO:0045184	Establishment of protein localization
GO:0008104	Protein localization
GO:0006886	Intracellular protein transport
GO:0034613	Cellular protein localization
GO:0070727	Cellular macromolecule localization
GO:0046907	Intracellular transport
Enrichment score: 3.6	
GO:0045454	Cell redox homeostasis
GO:0019725	Cellular homeostasis
GO:0042592	Homeostatic process
Enrichment score: 2.7	
GO:0006270	DNA replication initiation
GO:0006261	DNA-dependent DNA replication
GO:0006260	DNA replication
GO:0006259	DNA metabolic process

3.3. Methylation Analysis of the Zebrafish Genes Found to Have Altered Gene Expression in the DM and MM States. All members of the *DNA replication/DNA metabolism process* group found to have altered expression (upregulated) in the DM state and persisted in having altered expression (upregulated) in the MM state (Figure 3) were then analyzed as to the differential DNA methylation patterns observed between the control, DM, and MM states. Methylated DNA

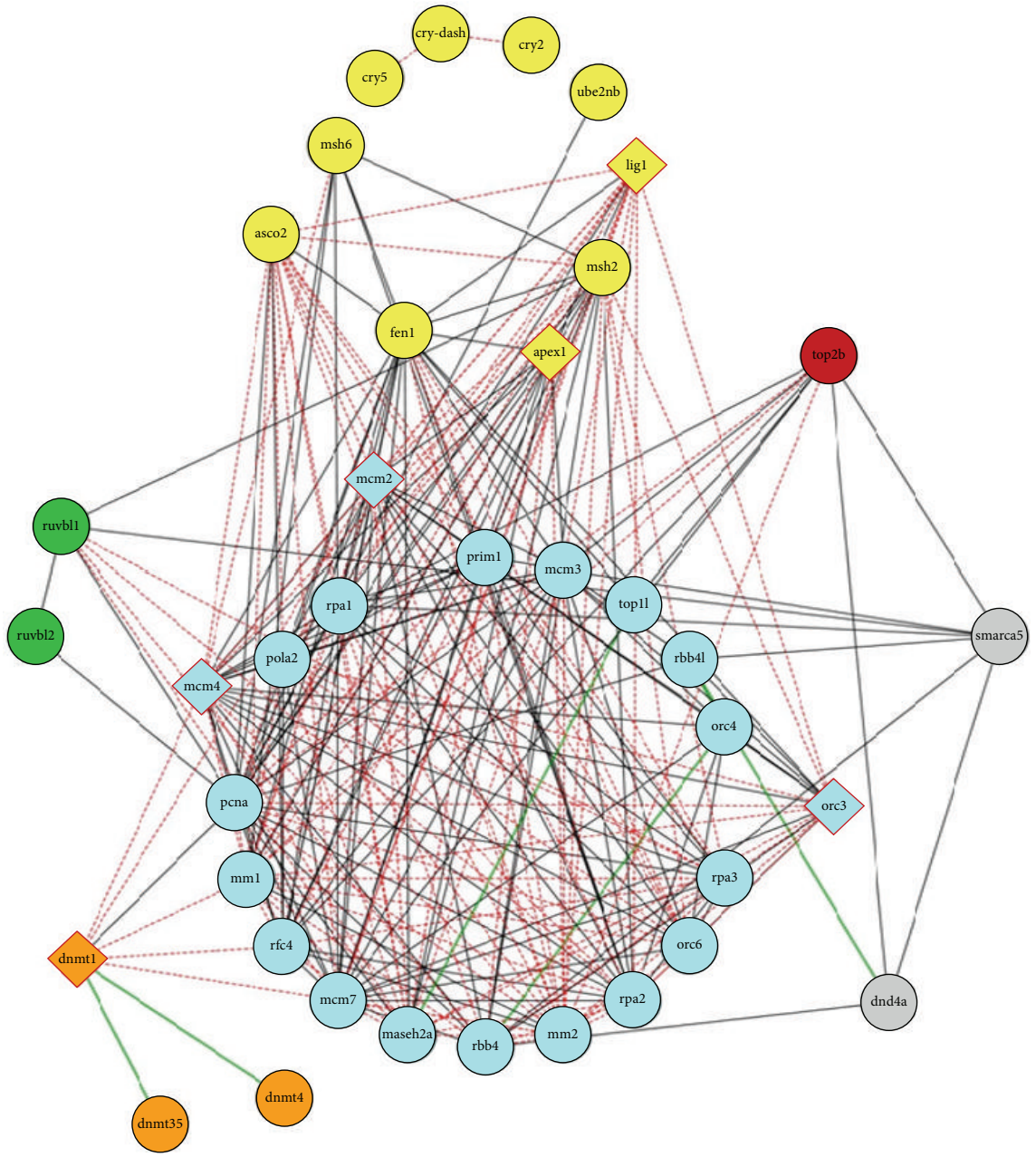

FIGURE 2: Gene network showing associations between genes that were differentially expressed in the DM and MM states relative to controls (genes of Figure 2 are listed in Supplemental Table 1). Nodes of the network represent genes that are grouped by their involvement in biological process as defined by the Gene Ontology and are colored accordingly (light blue denotes genes involved in DNA replication; gray, in chromatin remodeling; orange, in DNA methylation; green, in DNA recombination; yellow, in DNA repair; and brown, in DNA topological change). Diamond-shaped nodes represent genes with differentially methylated regions. Gene interactions confirmed experimentally and by coexpression are shown as black lines connecting nodes; red dotted lines show associations derived only from coexpression data; and green lines denote interactions confirmed by only experimental evidences.

regions are abbreviated as "MRs" in this paper. These genes were specifically analyzed in regard to (1) the position of MRs, (2) the percent GC content, and (3) the location of CpG islands relative to overall methylated content. In regard to the first point, our analysis found that MRs could be found (1) upstream of the transcription start site (TSS), (2) within the gene proper, and/or (3) downstream of the termination codon. The MRs for *dnmt1*, *mcm2*, and *orc3* are shown in Figure 4 and are depicted as red bars in that figure for the control, DM, and MM states.

In general, the trend for *dnmt1*, *mcm2*, and *orc3* was for MRs upstream of the TSS to be methylated in the normal state but to have a reduced methylated CpG content in the DM and MM states (MRs that are methylated in the normal state and

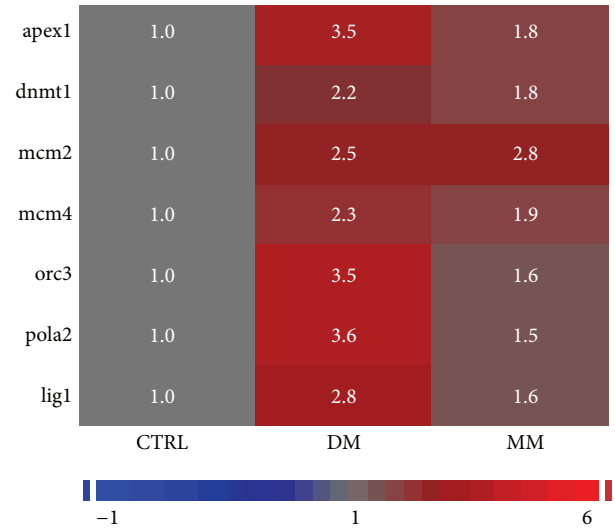

Figure 3: Heat map of expression of genes that are with altered expression in both the DM and MM condition and that are critical members of the *DNA replication/DNA metabolic* process group. As indicated, all genes listed were found to be upregulated.

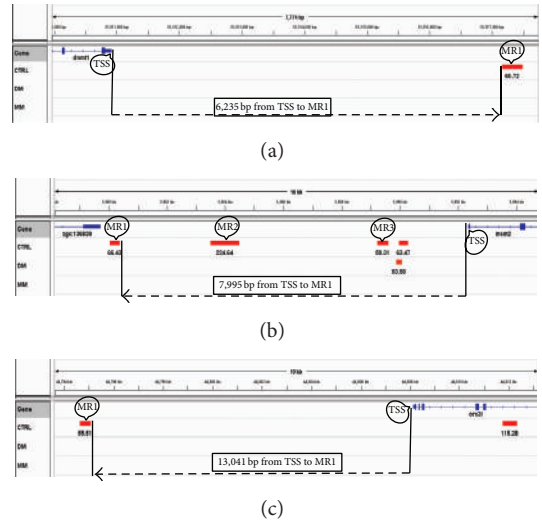

Figure 4: Methylated DNA regions (MRs) of zebrafish gene loci for *dnmt1* (a), *mcm2* (b), and *orc3* (c). Panels (a), (b), and (c) were prepared with IGV genome viewer. The scale on the top shows chromosomal coordinates. This program schematically represents the structure of the gene using conventional elements: (1) blue line represents introns and (2) blue blocks on the line represent exons. Circles with the "TSS" point to the transcription start site and the dashed arrow points in the direction of 5′ to 3′ of the DNA strand with the arrow pointing to the MR farthest upstream of the TSS as indicated for each diagram (bp distance indicated in the box above the dashed arrow). Three tracks separated by gray lines below gene track show localization of MRs for each of the three samples to include control (CTRL), diabetic state (DM), and metabolic memory state (MM). Methylated regions detected by the MACS algorithm are shown as red boxes. Those upstream of the TSS that are lost in both the DM and MM states are numbered (MR1, MR2, etc.) and shown within circles over the red bars. The sequence counts for MACS peaks are shown under each MR. (a) *dnmt1*, (b) *mcm2*, and (c) *orc3*. (a) *dnmt1*, chromosomal coordinates are chr3:53,494,972-53,510,920; (b) *mcm2*, chromosomal coordinates are chr22:3,992,357-4,013,603; and (c) *orc3*, chromosomal coordinates are chr17:44,806,126-44,836,951.

have a reduced methylated CpG content in both the DM and MM states are indicated by circles pointing to the MR with the gene's name listed under the gene proper (Figure 4)). We did find some exceptions to this trend for MRs within the gene proper. In the case of *dnmt1* (see Figure 4(a)), one methylated MR was found in normal tissue that was upstream of the TSS and this MR had a reduced methylated CpG content in the DM and MM states. For *mcm2* (Figure 4(b)), four MRs were identified and all four were upstream of the TSS. Three of the MRs upstream of the TSS were methylated in normal tissue but had a reduced methylated CpG content in both the DM and MM states, while the fourth upstream MR remained methylated in DM but had a reduced methylated CpG content methylated in MM. In the case of *orc3* (Figure 4(c)), one MR was found upstream of the TSS while another was within the gene proper. Both of these MRs were methylated in the normal state but had a reduced methylated CpG content in both the DM and MM states. MRs for *lig1, apex1, mcm4*, and *pola1* are shown in Figures 5 and 6.

The MRs for *lig1* and *apex1* are shown in Figure 5 and the MRs for *mcm4* and *pola1* are shown in Figure 6. The MRs for *mcm4* (Figure 6(a)) followed the trends shown for *dnmt1, mcm2*, and *orc3*. While each of these genes showed upregulation of their mRNA expression, there were variations in their methylation patterns as compared to *dnmt1, mcm2,* and *orc3*. In the case of apex1, there was a loss of MRs in the DM and/or MM states, but this occurred in the ORF and not upstream of the TSS. In contrast, *lig1* and *pola2* showed opposite DNA methylation patterns to that of the other genes. In their cases, *lig1* and *pola2* had no MRs in the control state while MRs appeared in the DM and/or MM states, indicating a hyperglycemia-induced hypermethylation which persisted into the MM state for *pola2*.

We then focused on representative members of the DNA replication/repair (*mcm2* and *orc3*) and DNA methylation (*dnmt1*) groups to conduct more detailed analysis of CpG methylation patterns within MRs (see Figure 7). This analysis involved application of the UCSC genome browser to identify canonical CpG islands (length > 200 bp, GC content > 50%, and $\text{Obs}_{\text{CpG}}/\text{Exp}_{\text{CpG}} > 0.60$) [25] and use of the EMBOSS software to find regions shorter than 200 bp with observed/expected CpG abundance ratio above 0.6 [25, 26]. This second type of less than 200 bp is more easily seen in Figure 7(b) for zebrafish *mcm2* (CpG island region indicated by the green bar in the figure). Many high CpG content regions associated with the zebrafish genes we studied could not be classified as being either canonical or of the second type, but these regions could be found to be methylated based on the zebrafish MeDIP sequencing data.

4. Discussion

This study was designed to investigate the underlying mechanisms that could explain the heritable nature of diabetic

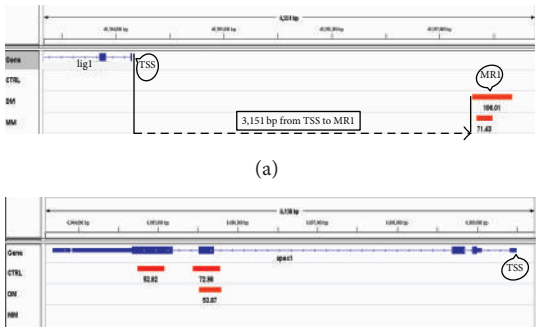

Figure 5: Methylated DNA regions (MRs) of zebrafish gene loci for *lig1* (a) and *apex1* (b). Panels (a) and (b) were prepared with IGV genome viewer. The scale on the top shows chromosomal coordinates. This program schematically represents the structure of the gene using conventional elements: (1) blue line represents introns and (2) blue blocks on the line represent exons. Circles with the "TSS" point to the transcription start site and the dashed arrow points in the direction of 5′ to 3′ of the DNA strand with the arrow pointing to the MR farthest upstream of the TSS as indicated for each diagram (bp distance indicated in the box above the dashed arrow). Three tracks separated by gray lines below gene track show localization of MRs for each of the three samples to include control (CTRL), diabetic state (DM), and metabolic memory state (MM). Methylated regions detected by the MACS algorithm are shown as red boxes. Those upstream of the TSS that have a loss of methylation in the DM and/or MM states are numbered (MR1, MR2, etc.) with the MACS peak ID numbers under each MR. (a) *lig1*, (b) *apex1*. The MRs of *apex1* are all located within the ORF and are not numbered, although they show a loss of methylation in the DM and/or MM as seen with *dnmt1*, *mcm2*, and *orc3*. In contrast *lig1* which did show upregulation in gene expression in the DM and MM states showed the opposite methylation pattern with the control state showing no MRs while the DM and MM states showed the appearance of MRs (Figure 5(a)).

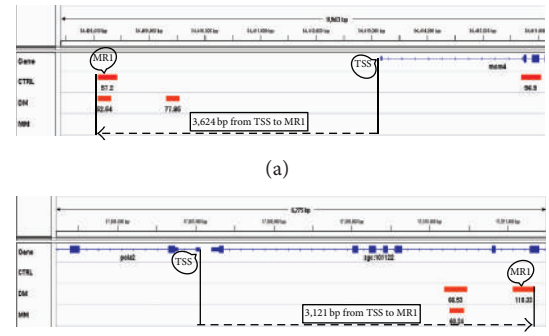

Figure 6: Methylated DNA regions (MRs) of zebrafish gene loci for *mcm4* (a) and *pola2* (b). Panels (a) and (b) were prepared with IGV genome viewer. The scale on the top shows chromosomal coordinates. This program schematically represents the structure of the gene using conventional elements: (1) blue line represents introns and (2) blue blocks on the line represent exons. Circles with the "TSS" point to the transcription start site and the dashed arrow points in the direction of 5′ to 3′ of the DNA strand with the arrow pointing to the MR farthest upstream of the TSS as indicated for each diagram (bp distance indicated in the box above the dashed arrow). Three tracks separated by gray lines below gene track show localization of MRs for each of the three samples to include control (CTRL), diabetic state (DM), and metabolic memory state (MM). Methylated regions detected by the MACS algorithm are shown as red boxes. MRs upstream of the TSS that have a loss of methylation in the DM and/or MM states are numbered (MR1, MR2, etc.) with the MACS peak ID numbers under each MR. (a) *mcm4*, (b) *pola2*. As shown in Figure 6(b), *pola2* mimicked *lig1*. Like *lig1*, *pola2* displayed upregulation of gene expression in the DM and MM states but showed no differential methylation pattern in the control state. Also like *lig1*, *pola2* was seen to have MRs detected in the DM and/or MM states.

metabolic memory. To this aim, gene enrichment analysis found a number of functional gene categories arising from our gene expression analysis of the control and DM fish. Of these functional gene categories we focused on the *DNA replication/DNA metabolism process* group because of its clear importance to the heritable nature of MM. While the other groups clearly contribute to the tissue dysfunctions observed in the DM and MM states, they do not explain the heritable basis of the pathology. When this group was analyzed in terms of transcripts that had an altered expression pattern in the DM group versus the control group, we found the genes listed in Supplemental Table 1. The validity of this list was enhanced when we determined, using gene network analysis, that these genes are interrelated based on current mouse and human literature. Of these genes, we found a subset whose altered expression pattern persisted into the MM state. These transcripts included genes related to (1) DNA replication/repair (*apex1, mcm2, mcm4, orc3,* and *pola2*) and (2) DNA methylation (*dnmt1*, a methyltransferase that functions during the replication process). We found that these genes showed a transcript upregulation pattern and all were found to have an altered methylation pattern associated with their loci (either upstream of the TSS or within the gene proper). As an extension of our previously published MeDIP global DNA methylation sequencing [3], we found that each of the genes with altered methylation patterns in the control, DM, and MM states was methylated upstream of its TSS in the control state but typically had a selective reduction of methylated CpGs in the DM state and this reduction in methylated CpGs was maintained in the MM state. The exception to this trend was *lig1* and *pola2* which both showed an increase in DNA methylation in the DM and MM states as compared to controls. This follows trends reported in the literature for DNA methylation changes in disease states, with hypomethylation being predominate over hypermethylation [27]. While any changes in DNA methylation patterns lead to an alteration in gene expression that subsequently leads to tissue dysfunctions [3, 17, 27], it is important to note that while increased methylation often results in decreased expression of the gene, this result is not always the case [28]. As indicated, this was the case for *lig1* and *pola2* in which an increase in gene expression was found in combination with increased methylation for the DM and/or MM states.

We also analyzed representative members of these two functional groups (*dnmt1, mcm2*, and *orc3*, as shown in Figure 7) in more detail to determine the specific CpG methylation patterns associated within the gene's loci. The reduction in DNA methylation of *dnmt1, mcm2*, and *orc3* occurred in

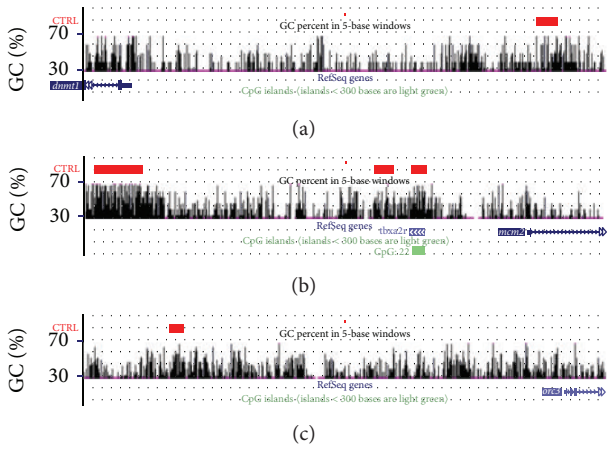

Figure 7: DNA methylation patterns within MRs of genomic areas upstream of the TSS for the *dnmt1*, *mcm2*, and *orc3* genes (Panels (a), (b), and (c), resp.). The zebrafish genes (and associated upstream genomic regions) were analyzed in regard to (1) methylated regions, (2) percent GC content, and (3) CpG islands. CpG islands are defined as containing at least 50% of CG dinucleotides within a region of at least 200 bp. As in Figure 4, red bars represent methylated regions (MRs) as defined by the MACS algorithm for the zebrafish genes and black vertical bars represent GC content in the selected genomic region. For *mcm2*, the three red bars represent MRs 1, 2, and 3 as depicted in Figure 4. Green bars (when present) indicate CpG islands (CpG island is a region with at least 200 bp and a GC percentage that is greater than 50% and with an observed-to-expected CpG ratio that is greater than 60%). Notice that the red bars overlay areas of elevated GC content that are not classified as canonical CpG islands except for the far right red bar for *mcm2*, as indicated by the lower green bar to the right of center in Panel 7(b).

CpG rich regions and/or CpG islands depending on the gene analyzed. In the case of zebrafish *mcm2* that contains multiple methylated regions (MRs), some of these MRs overlapped with CpG islands, while, in the case of *dnmt1* and *orc3*, CpG islands were not identified in MRs because the number of the CpG dinucleotide clusters was less than 200 in the regions upstream of the TSS (200 GC dinucleotides being the cut-off for a CpG island based on the criteria: length > 200 bp, GC content > 50%, and $\text{Obs}_{\text{CpG}}/\text{Exp}_{\text{CpG}} > 0.60$) [25]. This finding raises questions as to the actual relationship between CpG rich areas (those defined as CpG islands and those not defined as CpG islands) and the methylation process as pertaining to regulatory mechanisms affecting gene expression. It is interesting to note that methylation of CpG rich areas and CpG islands was restricted to a limited number of regions within the genomic area upstream of the TSS and was not seen in all areas that could have been methylated based on CpG content. How hyperglycemia induces this specific type of methylation is unknown but could be related to chromatin 3-dimensional structure. In this context, some CpG rich areas may be more accessible to the methylation machinery than other CpG rich areas in the same gene due to regional chromatin structural differences [29]. Moreover, recent studies related to histone-methylation indicate that higher levels of genomic control (termed, epigenetic control regions (ECRs) [30, 31]) exist for regulation of epigenetic processes [30, 31]. Whether such ECRs have a role in the epigenetic processes described in our studies remains to be determined.

As indicated in our previous 2012 article [3], not all genes in the zebrafish DM/MM model have methylation changes following hyperglycemia. Hyperglycemia is loci specific in terms of which genes are targeted for methylation changes. Does this mean that only genes with differential methylation patterns are susceptible to dysregulation of their expression? In this regard, it is important to note that network analysis indicates that genes of the DNA replication/repair group are all functionally interrelated. This means that changes in the methylation pattern of one particular gene would change that gene's expression and this changed gene expression pattern could in turn affect other genes that had no methylation changes associated with them because all genes in the network are functionally interconnected. Therefore a gene does not need to have an altered DNA methylation pattern for sustained upregulation or downregulation of its expression in the MM state. Evidence for this condition comes from the studies of Dehde et al. [32] who found that *pola2* has direct interactions with *mcm2* during DNA replication and therefore methylation changes in one can affect the other gene regardless of whether both genes have methylation changes. Compensatory expression mechanisms could trigger gene expression pattern changes in such a case. The regulatory effects of DNA methylation as related to changes in gene expression patterns are poorly understood and require additional study.

As an extension of the above discussion, it is important to note that many of the MRs we have identified in the DNA replication and repair genes are far upstream of each gene's transcription start sites. The functional importance of these MRs in the regulation of the genes we have identified has not been established in our studies and therefore, our data are correlative in this regard. Therefore, as an important next step in our studies, we will be applying molecular techniques that allow us to prevent MRs from being methylated *in vivo* so that we can then determine how this modification affects expression of the gene of interest. Prevention of methylation of the MR coupled with subsequent gene expression analysis (as compared to gene expression observed in the unmodified DM/MM state) will allow us to functionally tie MRs to specific genes. This approach will be the foundation for future studies using the zebrafish DM/MM model as well as accessible human tissues obtained from patients with diabetes (e.g., peripheral blood cells, exempt surgically disposed tissue).

The data obtained from the zebrafish model points the way to potential mechanisms in the human diabetic condition. In this regard and given the fact that, in comparison to the human genome, approximately 70% of human genes have at least one obvious zebrafish orthologue [33], the bioinformatics analysis applied to the zebrafish diabetic model may provide insight into the human disease state. This is also reinforced by the fact that zebrafish glucose regulation mimics that of all mammals to include the human. Moreover, reductions in DNA methylation regions can also be found in the genome of human diabetic patients [34]. In this particular case, hypomethylation in the promoter region

of the Connective Tissue Growth Factor (CTGF) gene has been reported for patients with T2 DM [34]. CTGF is a known regulator of cell proliferation as related to the process of angiogenesis and therefore is functionally tied to DNA replication and repair mechanisms.

In regard to the human diabetic state, it is well known that long term complications related to alterations in cell proliferation rates are characteristic of both type 1 and type 2 diabetic patients [35–40] and our current findings provide a molecular basis to help explain this type of tissue dysfunction due to changes in the transcription of genes fundamental to cell division. It should be noted that cell proliferation rate alterations as is seen in angiogenesis have been reported to include both increased and decreased cell division rates in both type 1 and type 2 DM, depending on the tissue studies [41]. Changes in cell proliferation rates have been highlighted in the zebrafish DM/MM model and these altered rates have been reported to affect a number of hyperglycemia-induced dysfunctional tissues of the model to include the CV system [4], visual system [2], and limb tissues [2, 3]. The data of the current study in the zebrafish DM/MM model indicate that future studies in human diabetic tissues should focus on DNA methylation changes in (1) genes that are susceptible to methylation changes and (2) genes that regulate cell proliferation to establish a potential basis for such deficits associated with the long term compilations seen in both type 1 and type 2 diabetic patients.

As a final note, it must be remembered that secondary complications in diabetes arise from a multitude of cellular, biochemical, and molecular factors as discussed by Brownlee over a decade ago [16] and more recently by Fowler [1] who focused on complications related specifically to the cardiovascular system. Even in the case of epigenetic mechanisms, DNA methylation is just one process among many (e.g., histone modifications and microRNA changes). Consequently, it is most likely that clinical approaches to the treatment and prevention of the secondary complications seen in diabetes will require multifaceted therapies, with strategies targeting DNA methylation changes being just one of many.

5. Conclusions

In conclusion, these studies provide a molecular basis for the many studies reporting altered cell proliferation rates in the long term diabetic condition. They point to DNA methylation changes and concomitant gene expression changes being tied to problems in DNA replication/repair genes, which are consistent with the alterations in cell proliferation observed in both the DM and MM states of the zebrafish DM/MM model. These observations may extend to the human diabetes conditions, where alterations in such genes as MCM2 have also been observed to occur [42]. Future studies will expand on these findings by translating that found in the zebrafish DM/MM genes to human genes whose DNA methylation patterns are altered in the diabetic state (such as those found with the human CTGF gene of patients with T2 diabetes). The ultimate aim will be to (1) determine that methylation changes in MRs are functionally tied to the genes of interest and (2) determine the effect of these changes on the ability of transcription factors to bind to their DNA binding sites. The proposed underlying mechanism in this latter case relates to transcription factor binding dysregulation via causing alterations in gene expression. These impairments in transcription factor binding would then lead to tissue dysfunction as observed in the long term disease.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Diabetic Retinopathy Is Strongly Predictive of Cardiovascular Autonomic Neuropathy in Type 2 Diabetes

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A well-established, comprehensive, and simple test battery was used here to re-evaluate risk factors for cardiovascular autonomic neuropathy (CAN) in type 2 diabetes. One hundred and seventy-four patients with type 2 diabetes were evaluated through the methods of deep breathing and Valsalva maneuver for correlation with factors that might influence the presence and severity of CAN. The Composite Autonomic Scoring Scale (CASS) was used to grade the severity of autonomic impairment, and CAN was defined as a CASS score ≥2. Results showed that nephropathy, duration of diabetes, blood pressure, uric acid, and the presence of retinopathy and metabolic syndrome significantly correlated with the CASS score. Age may not be a risk factor for diabetic CAN. However, the effects of diabetes on CAN are more prominent in younger patients than in older ones. Diabetic retinopathy is the most significant risk factor predictive of the presence of CAN in patients with type 2 diabetes.

1. Introduction

Cardiovascular autonomic neuropathy (CAN) is one of the most clinically relevant complications of diabetes. The risk of developing CAN in diabetes depends on several factors, the most intuitive and most well-established of which is chronic hyperglycemia, including the duration and glucose level. Old age, nephropathy, and vascular risk factors such as hypertension and dyslipidemia have also been associated with increased severity of CAN [1–7]. Identifying the risk factors of CAN is essential in providing important clues to its etiologies and can help physicians determine treatment guidelines.

The prevalence of CAN among patients with diabetes varies widely in different reports, perhaps due to different patient groups (different ages and different durations of diabetes), different tests used, and different diagnostic criteria [5, 6]. Although there are no uniform criteria or staging for diagnosing CAN, advances in autonomic laboratory testing in the past decades, especially with the introduction of noninvasive beat-to-beat blood pressure (BP) recording by Finapres [8], have greatly improved the sensitivity and specificity of evaluations of cardiovascular autonomic function [9]. The American Academy of Neurology has published a position paper on autonomic function tests [10]. The autonomic tests used by previous studies have significant limitations. For example, most of them focused on cardiovagal function, whereas adrenergic function was either omitted or evaluated simply by BP changes with postural change or handgrip [1, 2, 7, 11, 12]. Such methods may have limited sensitivity and specificity according to evidence-based assessment [10].

Furthermore, in some studies, absolute cut-off values are used to define autonomic "abnormalities." Thus, the confounding effects of age and sex are not eliminated.

The present study evaluated cardiovascular autonomic functions, including both cardiovagal and adrenergic functions, by using simple, time-saving, and well-established methods. Factors that might influence the presence and severity of CAN in patients with type 2 diabetes were also assessed. Lastly, the association between these risk factors and CAN was re-evaluated. The successful translation of these approaches to the clinics enables not only the prediction of outcome but also the assessment of the impact of factors on the therapeutic efficacy of patients with diabetes.

2. Patients and Methods

2.1. Inclusion and Exclusion Criteria. This cross-sectional study evaluated 174 patients with type 2 diabetes from the outpatient diabetes clinic at Kaohsiung Chang Gung Memorial Hospital between April 2011 and July 2011.

Patients were excluded if they had the following: (1) suffered from moderate-to-severe heart failure (NYHA class III and IV); (2) had any type of arrhythmia that prevented the analysis of heart rate variability, or pacemaker implantation due to any cause; (3) had neoplastic disorders; (4) had degenerative disorders known to affect the autonomic system, such as Parkinson's disease, diffuse Lewy-body disease, multiple system atrophy, and pure autonomic failure; or (5) had a history of major stroke (brain stem or large hemispherical lesions).

2.2. Study Protocol. The hospital's Institutional Review Committee on Human Research approved the study protocol, and all of the study subjects provided informed consent.

Each patient participated in a detailed interview regarding their personal disease and a physical examination that included measurements of height, weight, and waist circumference. All of the subjects then underwent an autonomic survey, including deep breathing and Valsalva maneuver (VM) tests, as described by Low [13].

2.3. Assessment of Cardiovascular Autonomic Function. Heart rate (HR) was derived from continuously recorded standard three-lead ECG (Ivy Biomedical, model 3000; Branford, CT). Arterial BP was continuously measured at the finger by using beat-to-beat photoplethysmographic recordings (Finometer Pro, Ohmeda; Englewood, OH). Parameters of HR response to deep breathing (HR_DB) and Valsalva ratio (VR) were obtained through tests computed by Testworks (WR Medical Electronics Company, Stillwater, MN). To quantify the degree of dysfunction, the measures of HR_DB and VR were transformed into normal deviates (NDs) by using the Neuropcentiles software (WR Medical Electronics Company) [14] and denoted by $Z_{\mathrm{HR\_DB}}$ and Z_{VR}, respectively.

The severity of CAN was assessed by using the cardiovagal and adrenergic subscores of the Composite Autonomic Scoring Scale (CASS) [15]. However, the scale was modified for the adrenergic subscore because the 5-minute head-up tilt test was not performed in the current study. Thus, the CASS version used here allotted 3 points instead of 4 for the adrenergic domain (Table 1).

Table 1: Modified Composite Autonomic Scoring Scale (subscores in cardiovagal and adrenergic domains).

	Cardiovagal
0	Normal
1	HR_DB mildly reduced but >50% of minimum
2	HR_DB reduced to <50% of minimum or HR_DB + VR reduced
3	Both HR_DB and VR reduced to <50% of minimum
	Adrenergic
0	Normal
1	Early phase II reduction >20 but <40 mmHg MBP (30–40 if >50 years) Late phase II does not return to baseline Pulse pressure reduction to ≤50% of baseline
2	Early phase II reduction >40 mmHg MBP
3	Early phase II reduction >40 mmHg + absent late phase II and phase IV

HR_DB: heart rate response to deep breathing; VR: Valsalva ratio; MBP: mean blood pressure.

2.4. Assessment of Risk Factors. The parameters evaluated were age, duration of diabetes, microvascular complications of diabetes (retinopathy and nephropathy), diabetic control (glycohemoglobin, HbA1c), associated medication (i.e., insulin, diuretics, beta-blockers, angiotensin-converting enzyme inhibitors/angiotensin II receptor blockers [ACEI/ARB], and calcium channel blockers [CCB]), inflammatory condition (hsCRP), body mass index (BMI), waist circumference, and biochemical data, including total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), uric acid, serum creatinine, and estimated glomerular filtration rate (GFR), which was calculated by using the modified Diet and Renal Disease equation. Albuminuria was determined by measuring the urinary albumin-to-creatinine ratio (UACR) in a spot urine test.

Retinopathy was determined through fundus photography by an experienced ophthalmologist (J.-J. Lee) who was blinded to the autonomic test results. Diabetic retinopathy (DR) was classified as one of the following three stages: stage 0: no apparent retinopathy (equivalent to the scale of Early Treatment of Diabetic Retinopathy Study [ETDRS] level 10); stage 1: nonproliferative diabetic retinopathy (NPDR; ETDRS level 20–55); and stage 2: proliferative diabetic retinopathy (PDR, ETDRS level >61) [16].

Moreover, hypertension was defined as a systolic BP > 140 mmHg and/or a diastolic BP > 90 mmHg, or being under antihypertensive treatment. Metabolic syndrome was defined as meeting at least two of the following criteria: (1) waist circumference >90 cm for men and >80 cm for women, (2) serum triglyceride level ≥150 mg/dL or being under drug treatment for elevated triglycerides, (3) serum HDL-C level <40 mg/dL in men and <50 mg/dL in women, or being

under drug treatment for low HDL-C, and (4) elevated BP (systolic BP ≥ 130 mmHg and/or diastolic BP ≥ 85 mmHg, or a previous diagnosis of hypertension).

2.5. Statistical Analyses. Data are expressed as mean ± SD or median (interquartile range) for continuous variables and as median (minimum, maximum) for ordinal variables. Associations between measurements were evaluated with Pearson correlation tests for normally distributed continuous data or with the Spearman nonparametric test for continuous data with skewness or for ordinal data. The chi-square test was used for analyses of dichotomous variables. Logistic regression analysis with the forward conditional method was used to identify the odds ratio (OR) of risk factor. Statistical significance was set at $p < 0.05$. All statistical analyses were conducted by using the IBM SPSS software package, version 17 (IBM, Inc., Armonk, NY).

3. Results

3.1. General Characteristics and Autonomic Function of Patients with Diabetes. Of the 174 (117 men, 57 women) patients diagnosed with type 2 diabetes, 56 were administered insulin therapy. Their demographic characteristics and biochemical and autonomic parameters are listed in Table 2. Most of them had hypertension (153/174) and metabolic syndrome (136/174). The histograms of cardiovagal and adrenergic subscores and CASS are shown in Figure 1. On the histogram, the total valid number of adrenergic subscores and CASS is <174 because subjects who had suboptimal Valsalva effort (expiratory pressure <30 mmHg or duration <10 s) and undetermined scores were not included in the analysis.

Of the 159 patients with a valid CASS score, 41.5% (66/159) had CAN, which was defined as a minimum score of 1 in both the cardiovagal and adrenergic domains or a minimum score of 2 in one domain [5, 17]. In other words, patients with a CASS score ≥2 were defined as having CAN.

Table 2: Characteristics and biochemical data of patients with type 2 diabetes.

Characteristics	Mean ± SD [median (IQR)]
Age (year)	63.8 ± 9.2
Male/female	117/57
Body weight (kg)	69.8 ± 12.2
Body height (cm)	162.4 ± 8.0
Body mass index (kg/m^2)	26.2 ± 3.7
Waist circumstance (cm)	93.2 ± 10.6
Duration of diabetes (year)	11.9 ± 7.0
HbA1c (mmol/mol) (NGSP, %)	55 ± 10 (7.2 ± 0.9)
GFR (mL/min/1.73 m^2)	60.4 ± 29.1
UACR (mg/mg)	0.10 [0.02, 0.38]
hsCRP (mg/L)	1.00 [0.44, 2.3]
UA (mg/dL)	7.3 ± 2.0
Cholesterol (mg/dL)	153.2 ± 29.5
LDL-C (mg/dL)	74.1 ± 26.3
HDL-C (mg/dL)	52.3 ± 13.6
Triglycerides (mg/dL)	114.0 [80.8, 168.0]
SBP (mmHg)	138.8 ± 19.1
DBP (mmHg)	74.2 ± 10.5
HR_DB (beats/min)	7.2 ± 4.6
VR	1.29 ± 0.18
Z_{HR_DB}	−1.04 ± 1.06
Z_{VR}	−1.83 ± 0.78
Cardiovagal subscore	0 [0, 3] [min, max]
Adrenergic subscore	0 [0, 2] [min, max]
CASS	0 [0, 5] [min, max]

n: valid case number; SD: standard deviation; IQR: interquartile range; HbA1c: glycohemoglobin; GFR: glomerular filtration rate; UACR: urinary albumin-to-creatinine ratio; UA: uric acid; hsCRP: high-sensitive C-reactive protein; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; SBP: systolic blood pressure; DBP: diastolic blood pressure; HR_DB: heart rate response to deep breathing; VR: Valsalva ratio; CASS: Composite Autonomic Scoring Scale.

3.2. Risk Factors Associated with CAN. The CAN group was younger (60.7 ± 9.4 versus 64.8 ± 8.3 years, $p = 0.005$) and had higher UACR levels (0.14 versus 0.05, $p = 0.001$), compared with the non-CAN group (Table 3). The CAN group had a significantly higher stage of DR than did the non-CAN group ($p < 0.001$). The proportion of patients using insulin and diuretics were borderline higher in the CAN group ($p = 0.050$ and 0.044, respectively). There was no difference between the two groups with respect to sex, BMI, waist circumference, diabetic profile (including duration of diabetes and HbA1c level), lipid profile, GFR, uric acid, or hsCRP level. Although the CAN group had a higher prevalence of metabolic syndrome and higher BP, the differences were not statistically significant.

Statistical analysis of the differences between clinical manifestations and laboratory data between the two patient groups revealed significant findings for the following parameters: age ($p = 0.005$), UACR ($p = 0.001$), insulin usage ($p = 0.05$), and stage of retinopathy ($p < 0.001$). The significant univariate factors and possible confounding factors used in stepwise logistic regression included age, UACR, insulin usage, and retinopathy stage. After analysis of all the above-mentioned variables, only age and retinopathy stage were independently associated with the presence of CAN. Each reduction of a year of age increased the rate of CAN by 5% ($p = 0.012$, adjusted OR = 0.95, 95% CI = 0.90–0.99). The patients with stage 1 and stage 2 DR had a higher risk of CAN than did those without CAN (stage 0) by adjusted ORs of 2.73 and 11.19, respectively (Table 4).

3.3. Associations between Risk Factors and Autonomic Parameters. An analysis of the association between risk factors and individual autonomic parameters and scores (Table 5) revealed that the correlations of age with HR_DB, cardiovagal subscore, and CASS were significant. The duration of diabetes and systolic BP both significantly correlated with Z_{VR}. As for the indicators of nephropathy, UACR significantly correlated with all autonomic parameters and scores, whereas GFR correlated with only some of them. There were significant correlations between UA and adrenergic subscores and between

Table 3: Demographic data between groups of CAN and non-CAN.

	Non-CAN ($n = 93$)	CAN ($n = 66$)	p value
Age (year)	64.8 ± 8.3	60.7 ± 9.4	**0.005****
Body mass index (kg/m^2)	26.0 ± 3.6	26.3 ± 3.8	0.524
Waist circumstance (cm)	92.9 ± 10.6	92.9 ± 10.8	0.815
Duration of diabetes (year)	11.5 ± 6.7	12.8 ± 7.5	0.331
SBP (mmHg)	137.1 ± 20.1	141.0 ± 18.7	0.205
DBP (mmHg)	74.4 ± 11.3	74.5 ± 10.0	0.921
HbA1c (mmol/mol) (NGSP, %)	54 ± 9 (7.1 ± 0.8)	56 ± 12 (7.3 ± 1.1)	0.175
GFR (mL/min/1.73 m^2)	63.1 ± 26.8	58.9 ± 31.9	0.120
UACR (mg/mg)	0.05 [0.01, 0.24]	0.14 [0.05, 0.57]	**0.001****
hsCRP (mg/L)	0.95 [0.44, 2.02]	1.05 [0.49, 2.30]	0.442
UA (mg/dL)	7.1 ± 1.8	7.6 ± 2.3	0.126
Cholesterol (mg/dL)	151.3 ± 32.8	155.7 ± 25.0	0.115
LDL-C (mg/dL)	72.7 ± 28.9	76.3 ± 23.8	0.112
HDL-C (mg/dL)	51.8 ± 12.6	54.7 ± 14.8	0.222
Triglycerides (mg/dL)	135.0 ± 85.0	123.5 ± 70.0	0.600
Sex (F/M)	26/67	24/42	0.263
Insulin	24/93	27/66	**0.050***
ARB/ACEI	72/93	54/66	0.678
Beta-blocker	24/93	27/66	0.055
CCB	34/93	29/66	0.410
Diuretics	46/93	44/66	**0.044***
Metabolic syndrome	66/93	55/66	0.059
Retinopathy†			<**0.001****

HbA1c: glycohemoglobin; GFR: glomerular filtration rate; UACR: urinary albumin-to-creatinine ratio; hs-CRP: high-sensitive C-reactive protein; UA: uric acid; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; ARB: angiotensin receptor blocker; ACEI: angiotensin-converting-enzyme inhibitor; CCB: calcium-channel blocker.

*$p < 0.05$; **$p < 0.01$.

†Retinopathy was categorized into stages 0, 1, and 2.

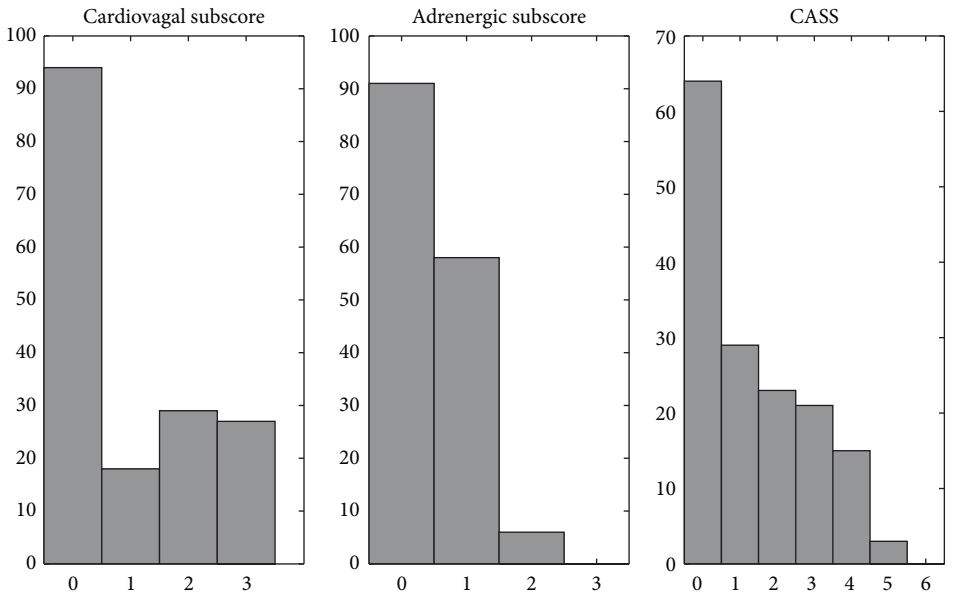

Figure 1: Histograms of cardiovascular autonomic scores, including cardiovagal and adrenergic subscores, and cardiovascular CASS score of the study patients. CASS: Composite Autonomic Scoring Scale.

Table 4: Logistic regression analyses of risk factors for cardiovascular autonomic neuropathy.

	Adjusted OR of CAN (95% CI)	p value
Age	0.95 (0.90–0.99)	**0.012***
UACR	1.24 (0.66–2.33)	0.504
Retinopathy		
Stage 1	2.73 (1.14–6.54)	**0.024***
Stage 2	11.19 (4.15–30.16)	<**0.001***

OR: odds ratio; CAN: cardiovascular autonomic neuropathy; CI: confidence interval; UACR: urinary albumin-to-creatinine ratio.
$^*p < 0.05$.

UA and CASS. The presence of DR and metabolic syndrome was significantly associated with lower Z_{HR_DB} and Z_{VR}.

4. Discussion

Although cardiovascular autonomic reflex tests [10] have been accepted as the gold standard for the evaluation of autonomic function, no unanimous criteria for diagnosis of CAN have been adopted to date [5, 6]. This study intended to introduce a simple and time-saving test battery that can be applied to clinical practice, rather than just study purposes. These test procedures can be performed within 10–15 min, and both cardiovagal and adrenergic functions can be evaluated. The deep breathing test was previously recommended as an optimal test for cardiovagal function [10]. VM tests can detect adrenergic failure with greater sensitivity than can orthostatic BP recordings [9]. Furthermore, these methods have well developed age normative data and a corresponding scaling score for the degree of severity.

The prevalence of CAN in the study patients was 41.5% (66/159). Among them, 44 (66.7%) had a CASS score of 2 or 3. The prevalence of CAN in previous reports varied widely from 2.5 to 50% [6]. Factors that influence the prevalence of CAN include the diagnostic criteria used, patient age, and the duration of diabetes. However, our results are consistent with findings in most previous reports in the aspect that autonomic neuropathy is common in diabetes, although it tends to be of a mild severity [3, 17].

Surprisingly, the CAN group was younger than the non-CAN group. There were strongly significant correlations between age and Z_{HR_DB} and between age and the cardiovagal subscore. However, the correlation coefficient for the correlation between age and Z_{HR_DB} was positive, whereas that for the correlation between age and the cardiovagal subscore was negative, indicating that older age is associated with larger ND (and less abnormality). In addition, in the multivariate analysis, the OR for CAN was 0.94 for age, suggesting that CAN is less likely to occur in older age. These findings seem to contradict those in several previous reports showing that age is a risk factor for CAN [1, 18]. Those studies used absolute cut-off values as the definition of "abnormal" autonomic function and thus the confounding effects of age were not eliminated. However, according to the report of expert panels on consensus of diabetic neuropathy in Toronto, age normative values should be used in testing cardiovascular autonomic function since age is the most important cofounding factor [5, 6]. In fact, in our data, if the original measures were used instead of the Z-scores for association analyses, the correlation coefficient would have been negative. The method of using percentiles or NDs (Z-scores) to express the degree of test abnormalities was introduced by Dyck et al. [14]. This statistical method gives useful information about dysfunction or disease even when results fall within the range of normal values. In addition, the age (and gender) effects can be eliminated by using the transformed Z-scores. Because the current study focused on CAN in diabetes, the effects of normal aging on the autonomic system had to be excluded. Thus, using Z_{HR_DB} and Z_{VR} was more appropriate than using HR_DB and VR in the analyses. The results here are not unique since the findings by O'Brien et al. are similar [4]. Using age-adjusted normal ranges rather than absolute cut-off values in this study, abnormal autonomic scores correlated significantly with the duration of diabetes but not with age. In addition, the frequency of abnormal autonomic scores was greatest in the group aged 40–49 years rather than in the oldest group. The Toronto consensus panel in 2009 did not include old age as a risk factor of CAN [6]. Furthermore, two previous studies found that a younger age of onset is a risk factor for diabetic retinopathy [19] and nephropathy [20]. Considering that diabetic retinopathy, nephropathy, and neuropathy share a common mechanism, that is, microvasculopathy, our results may not be unexpected. We suggest that the duration of undiscovered type 2 diabetes, which may be longer in younger patients, may contribute, at least partially, to the phenomenon. Overall, the current results suggest that the effects of diabetes on cardiovascular autonomic function are more obvious in younger patients than in older ones; however, it is impossible to demonstrate in detail the influence of age on diabetic CAN through only a cross-sectional study. Elucidating the real effects of age on diabetic CAN warrants further longitudinal cohort studies.

According to our data, DR is a strong predictor for CAN. The importance of such a finding has not been sufficiently stressed although the correlation between CAN and retinopathy has been mentioned in some reports [2, 21, 22]. Schmid et al. found that proliferative DR was related to CAN in type 2 diabetes [22]. However, their case number was limited (n = 17 and 18 for non-CAN and CAN groups, respectively) and thus the results may be less compelling. The results here corroborate such findings and suggest that fundus photography may be an alternative to autonomic function testing in hospitals where facilities for the latter test are unavailable, because of the robust ORs (2.73 and 11.19 for stage 1 and stage 2, respectively).

The correlation between nephropathy and CAN has been reported in several studies [23–25]. The results are consistent with those of previous findings. Although in multivariate logistic analysis, UACR is not a significant predictor for CAN, there are strong correlations between UACR and each autonomicz parameter in bivariate analyses. Albuminuria is often

Table 5: Univariate correlation analysis between individual risk factors and autonomic parameters/scores.

	Z_{HR_DB}	Z_{VR}	Cardiovagal subscore	Adrenergic subscore	CASS
Age	**0.390****	0.004	**−0.371****	0.158	**−0.176***
Body mass index	−0.107	−0.079	0.096	0.003	0.085
Waist circumstance	−0.071	−0.049	0.056	−0.077	0.029
Duration of diabetes	−0.017	**−0.200***	0.023	0.154	0.077
HbA1c	−0.089	−0.077	0.145	−0.022	0.069
SBP	−0.020	**−0.205***	0.051	0.148	0.081
DBP	0.007	−0.113	−0.039	0.013	−0.050
GFR	0.086	**0.209***	−0.048	**−0.287****	−0.153
UACR	**−0.282****	**−0.287****	**0.289****	**0.225****	**0.326****
hsCRP	−0.088	−0.153	0.093	0.106	0.102
UA	−0.090	−0.139	0.111	**0.248****	**0.187***
Cholesterol	−0.117	−0.078	0.122	0.028	0.073
LDL	−0.109	−0.063	0.111	0.045	0.092
HDL	−0.028	0.025	0.035	0.022	0.022
Triglycerides	0.029	−0.146	0.011	0.042	0.024
Retinopathy†	**−0.429****	**−0.346****	**0.435****	**0.248****	**0.429****
Metabolic syndrome	−0.095	**−0.200***	**0.164***	**0.172***	**0.203***

HbA1c: glycohemoglobin; SBP: systolic blood pressure; DBP: diastolic blood pressure; GFR: glomerular filtration rate; UACR: urinary albumin-to-creatinine ratio; hs-CRP: high-sensitive C-reactive protein; UA: uric acid; LDL: low-density lipoprotein; HDL: high-density lipoprotein.
$^{*}p < 0.05$; $^{**}p < 0.01$.
†Retinopathy is categorized into stages 0, 1, and 2.

considered as a manifestation of microvasculopathy. The strong correlation between UACR and autonomic function again supports the notion that microvasculopathy plays an important role in CAN. Although albuminuria and decreased GFR are both considered indicators of nephropathy, the results here show that the correlation between CAN and UACR may be stronger than that between CAN and GFR. A previous study by Sterner et al. showed that a significant correlation between albuminuria and low GFR only exists in patients with type 1 diabetes but not in those with type 2 [26]. The more complicated and heterogeneous pathophysiology of reduced GFR in type 2 diabetes compared to that in type 1 diabetes may explain such findings.

Vascular risk factors such as hypertension and dyslipidemia have been associated with CAN. The data here shows that systolic BP significantly correlates with Z_{VR}; however, the interaction is likely to be reciprocal rather than unidirectional. Although hypertension may contribute to the existence of CAN, causing the decreased Z_{VR} that usually suggests blunted baroreflex sensitivity, patient with blunted baroreflex sensitivity tend to be hypertensive [27]. There was no significant correlation between lipid profile and cardiovascular autonomic function in this study.

The effects of UA on autonomic function or cardiovascular function remain controversial [28, 29], although there were significantly positive correlations between UA level and adrenergic subscore in this study. There was a borderline difference in insulin use between the CAN and non-CAN groups, which may be explained by confounding factors. The patient group with insulin treatment tended to have longer DM duration, higher HbA1c, and poorer renal function.

This study has some limitations. First, the prevalence of CAN in such patients cannot reflect the real conditions of general patients with diabetes. Patients with better compliance tend to be recruited in studies; hence, these patients have relatively good serum glucose control. The HbA1c value of the patients in this study was 7.2 ± 0.9. This narrow HbA1c spectrum may explain why statistical analyses fail to show any significant correlation between HbA1c and autonomic parameters. Furthermore, the medication effects on autonomic function tests were not eliminated in this study. Beta-blockers, CCBs, and diuretics are likely to influence the autonomic test results. Due to ethical considerations, these drugs were not stopped before the tests. Fortunately, the effects did not seem to be obvious and there was only a borderline significant difference in diuretic use between the CAN and non-CAN groups.

In conclusion, retinopathy is the most significant risk factor in predicting the presence of CAN in patients with type 2 diabetes. Old age may not be a risk factor for diabetic CAN. On the contrary, the effects of diabetes on CAN are more prominent in younger patients than in older ones.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Drs. Cheng-Hsien Lu and Rue-Tsuan Liu contributed equally to this work.

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