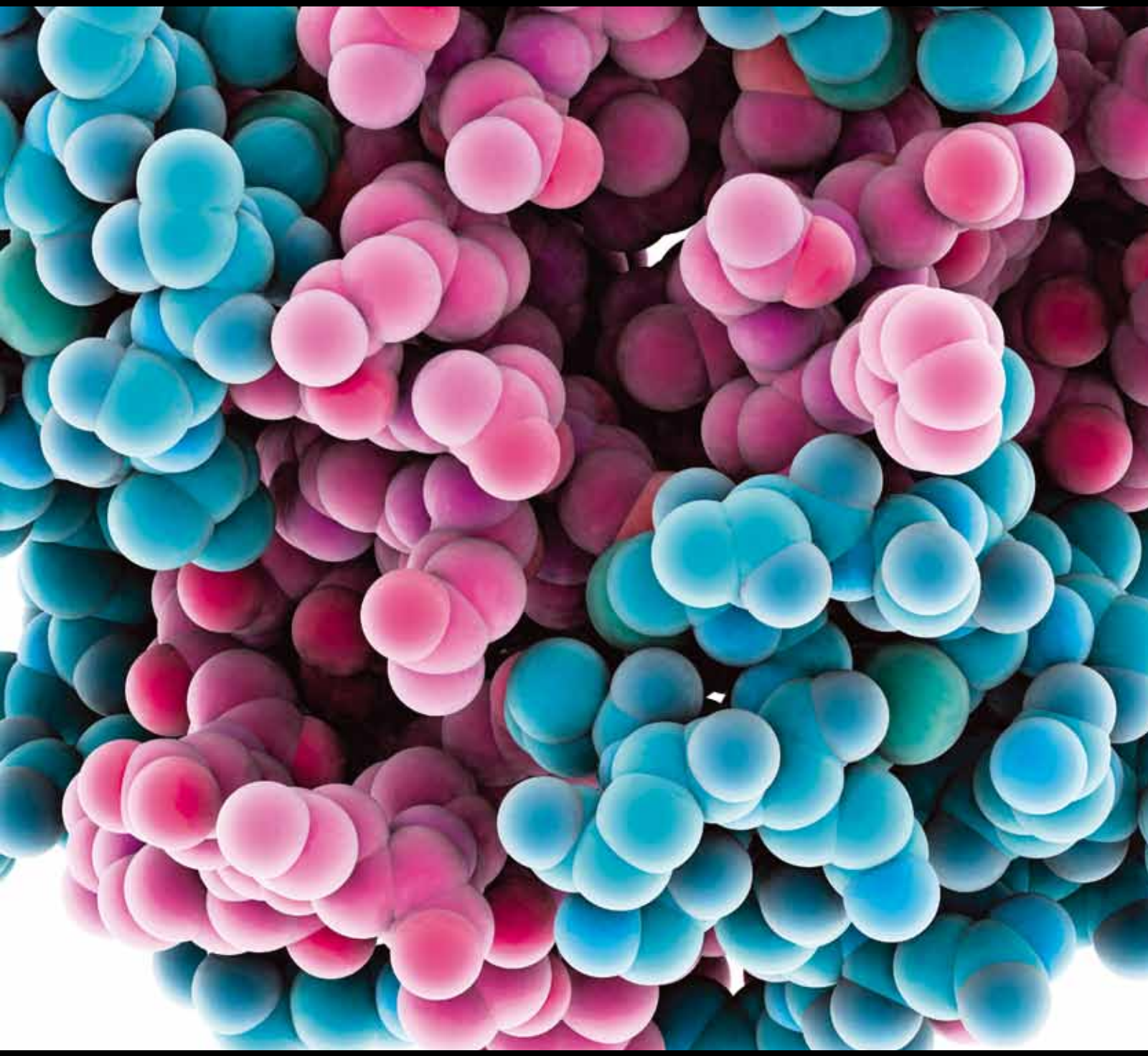
Journal of Diabetes Research

Complications of Diabetes

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



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Editorial Board

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Editorial
Complications of Diabetes

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Diabetes is justly recognized as an emerging global epidemic, representing one of the leading causes of morbidity and mortality worldwide. Hyperglycemia, the common characteristic of both type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM), has the potential to cause serious complications due to its insidious and chronic nature. The present special issue has been designed to publish original and review articles highlighting recent fundamental advances in our understanding of diabetic complications. Emphasis has been given on the underlying molecular mechanisms, the new technologies that have been introduced to facilitate early diagnosis, and the new potential therapies for these complications. There are 30 articles in total, which cover 6 thematic areas: pathogenesis of diabetic complications, diabetic neuropathy, nephropathy, retinopathy, macrovascular complications, and miscellaneous complications.

(a) Pathogenesis of Diabetic Complications. There is growing evidence that the underlying mechanisms in the pathogenesis of diabetic complications include oxidative stress created by the overproduction of reactive oxygen species (ROS) and defects in the insulin signal transduction pathway in which ceramide, a bioactive sphingolipid, may have an important inhibitory effect [1, 2].

In a paper of this special issue entitled "Pathogenesis of Chronic Hyperglycemia: From Reductive Stress to Oxidative Stress," L.-J. Yan reviews the role of reductive stress in the generation of oxidative stress. This review provides an interesting and novel approach on the pathogenesis of diabetic complications, emphasizing the importance of reductive stress in this complex process.

Another article included in this special issue by M. Mihailović et al. entitled "Protective Effects of the Mushroom *Lactarius deterrimus* Extract on Systemic Oxidative Stress and Pancreatic Islets in Streptozotocin-Induced Diabetic Rats" describes a study designed to assess the effects of mushroom *Lactarius deterrimus* extract on pancreatic β-cells and on systemic oxidative stress. This study showed that diabetic rats treated with the abovementioned extract exhibited better glycemic profile and a remarkable increase in β-cell mass.

In the same spirit, J.-F. Hu et al. in their paper titled "Ethanol at Low Concentration Attenuates Diabetes Induced Lung Injury in Rats Model" provide some evidence that ethanol at low concentration may prevent hyperglycemia-induced oxidative stress injury of lungs in diabetic rats. However, these results have been derived from rats, and, therefore, new studies should be designed in humans to explore such potential favorable effects.

The final paper of this thematic area is of M. E. Hansen et al. entitled "Insulin Increases Ceramide Synthesis in Skeletal Muscle." This study highlights the key role of insulin on ceramide metabolism in skeletal muscle and suggests that there might be an insulin-induced augmentation in the expression of genes controlling ceramide biosynthesis. Obviously, these results are promising and may help towards defining a novel therapeutic target.

(b) Diabetic Neuropathy. One of the most common complications of diabetes is diabetic peripheral neuropathy (DPN). Moreover, not only can chronic hyperglycemia have various negative effects on the central nervous system, but it can also cause gastroparesis [3, 4]. The role of glycemic variability (GV), which is recognized as an important component of the overall glycemic control, in diabetic neuropathy is also under investigation [5].

The paper entitled "Relationship between Autonomic Nervous System Function and Continuous Interstitial Glucose Measurement in Patients with Type 2 Diabetes" by S. Kalopita et al. aimed to examine if there is an association between the function of ANS and GV in patients with T2DM. This study showed that heart rate variability (HRV) was inversely associated with GV. Further prospective studies are required to ascertain the role of GV in the pathogenesis of ANS dysfunction.

In their paper titled "Decreased Neuronal Bursting and Phase Synchrony in the Hippocampus of Streptozotocin Diabetic Rats," Z. Qiao et al. explored the effects of an active fragment of amyloid precursor protein (APP 17-mer peptide) on the function of the hippocampus in streptozotocin-diabetic rats. Authors found that treatment with APP 17-mer peptide partially reversed the effects of diabetes on parameters of burst activity. These findings provide a more complete picture on the effects of diabetes on the function of hippocampus and encourage further enquiry into the changes of neuronal function in diabetes.

The study entitled "The Influence of Peripheral Neuropathy, Gender, and Obesity on the Postural Stability of Patients with Type 2 Diabetes Mellitus" by A. Herrera-Rangel et al. evaluated postural stability in patients with T2DM. The authors demonstrated that measures of postural stability were positively associated with the presence of peripheral neuropathy. These results shed light on the role of diabetes in the development of balance disorders.

Turning their attention to biomarkers of early nerve damage, A. Sun et al. in their paper titled "Urinary Methylmalonic Acid as an Indicator of Early Vitamin B_{12} Deficiency and Its Role in Polyneuropathy in Type 2 Diabetes" examined the levels of urinary methylmalonic acid (uMMA) in Chinese patients with T2DM and its relationship with serum vitamin B_{12} and polyneuropathy. The authors claimed that uMMA could be used as a sensitive marker of early polyneuropathy in patients with diabetes. However, the underlying mechanism of this relationship is unknown and needs to be further addressed.

The paper entitled "Vomiting and Dysphagia Predict Delayed Gastric Emptying in Diabetic and Nondiabetic Subjects" by D. Boltin et al. investigated the predictors of delayed gastric emptying in patients with and without diabetes. This study provides a more complete picture on gastric emptying procedure and how it is affected by diabetes.

E. Søfteland et al. in their paper titled "Rectal Sensitivity in Diabetes Patients with Symptoms of Gastroparesis" documented that patients with diabetes and gastroparesis exhibited reduced rectal sensitivity and impaired heart rate variability, as compared with healthy volunteers. As the authors suggest, the evaluation of heart rate variability could be developed into a rational screening test for diabetic gastroparesis. This is a promising hypothesis that needs to be confirmed by further studies.

X. Wang et al. in their article entitled "Early Detection of Atrophy of Foot Muscles in Chinese Patients of Type 2 Diabetes Mellitus by High-Frequency Ultrasonography" examined the efficacy of ultrasonography in detecting foot muscle atrophy in Chinese T2DM patients. The study showed that indices of muscular atrophy were significantly lower in patients with DPN. The clinical implication of this study is that high-frequency ultrasonography could be used to increase the timely detection of foot muscle atrophy in T2DM patients even before DPN becomes manifest.

S. L. Misra et al. in the paper of this thematic area entitled "Peripheral Neuropathy and Tear Film Dysfunction in Type 1 Diabetes Mellitus" explored the relationship between peripheral neuropathy and the quality of ocular surface in patients with T1DM. The findings suggest that ocular surface abnormalities develop in parallel with DPN and the two conditions are strongly connected to each other. Clearly, there is a lot to learn about this issue in the future.

(c) Diabetic Nephropathy. Diabetic nephropathy (DN) is the major cause of end-stage renal disease, although its pathogenesis is not fully understood. Emerging evidence suggests that epigenetic modifications and some microRNAs may play a role in the pathogenesis of DN by altering the expression of several genes and controlling certain intracellular pathways [6, 7].

The paper entitled "The Role of MicroRNAs in Diabetic Nephropathy" by H. Wu et al. provides an overview of the potential involvement of these small noncoding RNAs in DN. This comprehensive review leads to a more thorough understanding of the pathogenesis of DN at the molecular level. It may be hoped that some of these molecules may, in the foreseeable future, serve as potential targets for pharmaceutical intervention to prevent or at least delay DN.

In their review article entitled "Histone Lysine Methylation in Diabetic Nephropathy," G. Sun et al. discuss in detail the role of histone methylation in the pathogenesis and progression of DN and summarize recent advances in the field of epigenetic modifications of genes. All in all, histone methylation and other epigenetic modifications of DNA open up a new vista in our understanding of how DN can develop under certain interference between genes and environment.

(d) Diabetic Retinopathy. Nowadays, the importance of adequate glycemic control in the prevention of diabetic retinopathy (DR) is well established [8]. However, other factors may play a role in the pathogenesis of this complication of diabetes. There are an increasing number of studies on the associations of DR with various polymorphisms of genes such as vascular endothelial growth factor (VEGF) and eNOS gene [9, 10].

L. Han et al. in their paper titled "The Associations between VEGF Gene Polymorphisms and Diabetic Retinopathy Susceptibility: A Meta-Analysis of 11 Case-Control Studies" performed a comprehensive meta-analysis to evaluate the relationship between 5 VEGF gene polymorphisms and

susceptibility to DR. Significant associations were found between DR susceptibility and two out of five VEGF gene polymorphisms. These findings add to the growing body of literature on the role of VEGF polymorphisms in DR risk.

Another meta-analysis by Z. Ma et al. in the paper entitled "Association between eNOS 4b/a Polymorphism and the Risk of Diabetic Retinopathy in Type 2 Diabetes Mellitus: A Meta-Analysis" showed a lack of association between eNOS-4b/a polymorphism and the risk of DR. Although this meta-analysis was based mainly on Asian populations, it provides a better understanding of the relationship between eNOS-4b/a gene polymorphisms and DR.

Y. Lin et al. in their paper entitled "Serum Fibroblast Growth Factor 21 Levels Are Correlated with the Severity of Diabetic Retinopathy" found higher fibroblast growth factor 21 (FGF21) concentration in patients with diabetic retinopathy. These findings are used as evidence for a potential role of FGF21 in the pathogenesis of diabetic retinopathy. However, larger prospective studies are needed to establish this relationship.

Focusing on T1DM, S. B. Polat et al. in their article entitled "Evaluation of Serum Fibrinogen, Plasminogen, α2-Anti-Plasmin, and Plasminogen Activator Inhibitor Levels (PAI) and Their Correlation with Presence of Retinopathy in Patients with Type 1 DM" compared serum fibrinogen, plasminogen, α2-anti-plasmin, and PAI levels in T1DM patients with different degrees of DR and in healthy voluntaries. Subjects with T1DM had significantly higher α2-anti-plasmin levels, and these levels exhibit a positive correlation with HbA_{1c}. Clearly, this study adds new useful information and leads towards a better understanding of the fibrinolytic and thrombotic status in the pathogenesis of DR in T1DM.

In their paper entitled "Vitamin Status as a Determinant of Serum Homocysteine Concentration in Type 2 Diabetic Retinopathy," P. Fotiou et al. found that patients with DR exhibited higher serum homocysteine and lower vitamin B_{12} and serum folic acid levels, as compared with those who did not have DR. Based on their findings, the authors claimed that hyperhomocysteinemia, especially when accompanied by low folic acid and vitamin B_{12} levels, can serve as an independent risk factor for DR.

A. Praidou et al. in the paper entitled "Diabetic Retinopathy Treated with Laser Photocoagulation and the Indirect Effect on Glycaemic Control" reported that the patients with DR who had been treated with laser photocoagulation showed better glycemic control compared with untreated patients with DR. These unexpected findings suggest that treatment of DR with laser photocoagulation might have a psychological effect on patients, motivating them to improve their lifestyle and their adherence to treatment.

Z. Torok et al. in their paper entitled "Combined Methods for Diabetic Retinopathy Screening, Using Retina Photographs and Tear Fluid Proteomics Biomarkers" aimed to develop a novel automated method for DR screening. The authors ended up with a combined screening method for detection of microaneurysms, which resulted in a significant improvement of both sensitivity and specificity. Taken together, these findings raise hope for a more promising and accurate diagnostic modality for DR.

(e) Macrovascular Complications. Prior studies have proposed that measuring biomarkers of atherosclerosis can be helpful in detecting macrovascular complications of diabetes, such as subclinical carotid disease [11].

The clinical study described in the paper entitled "Relationship between HgbA1c and Myocardial Blood Flow Reserve in Patients with Type 2 Diabetes Mellitus: Noninvasive Assessment Using Real-Time Myocardial Perfusion Echocardiography" by R. Huang et al. aimed to scrutinize the relationship between HbA_{1c} and myocardial perfusion in patients with T2DM. The study showed that optimal glycemic control is associated with a preservation of myocardial blood flow reserve (MBFR) in T2DM patients who are at risk for CAD. Undeniably, this study provides robust clinical evidence that macrovascular complications of diabetes can be prevented by early and tight glycemic control.

The article entitled "Cumulative Effects of Hypertension, Dyslipidemia, and Chronic Kidney Disease on Carotid Atherosclerosis in Chinese Patients with Type 2 Diabetes Mellitus" by C. Yuan et al. showed that patients with multiple risk factors for atherosclerosis exhibit higher carotid plaque score, as compared with those having fewer risk factors. This work adds to the growing body of literature on the utility of risk factors estimated by ultrasound for the diagnosis and prognosis of carotid atherosclerosis in T2DM patients.

M. Celik et al. in their work entitled "Relation of Asymmetric Dimethylarginine Levels to Macrovascular Disease and Inflammation Markers in Type 2 Diabetic Patients" reported that serum asymmetric dimethylarginine (ADMA) levels were significantly higher in patients with T2DM and macrovascular complications. These findings are important because they highlight the potential role of ADMA in the pathogenesis of atherosclerosis in T2DM.

J.-M. Chen et al. in their work titled "Acarbose Treatment and the Risk of Cardiovascular Disease in Type 2 Diabetic Patients: A Nationwide Seven-Year Follow-Up Study" used nationwide insurance claim dataset to scrutinize if there is a benefit of acarbose treatment on cardiovascular disease (CVD) in T2DM. The authors found that patients receiving acarbose showed a lower risk of developing CVD, despite the fact that they exhibited a higher hazard ratio for CVD during the first 12 months of this treatment. These findings ensure a more thorough view of the protective role of acarbose in the cardiovascular system.

(f) Miscellaneous Complications. Diabetic foot is a major complication of diabetes characterized by the presence of chronic ulcers that often fail to heal. In the pathogenesis of chronic ulcers, matrix metalloproteinases may play a pivotal role [12]. L. Li et al. in their study entitled "The Effect of Autologous Platelet-Rich Gel on the Dynamic Changes of the Matrix Metalloproteinase-2 and Tissue Inhibitor of Metalloproteinase-2 Expression in the Diabetic Chronic Refractory Cutaneous Ulcers" examined the effect of autologous platelet-rich gel (APG), used as treatment of diabetic chronic refractory cutaneous ulcers, in the expression of matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinases (TIMPs), and transforming growth factor- (TGF-) β1. This study enriches our knowledge on the

beneficial effect of APG on recalcitrant diabetic cutaneous wound healing.

Statins are commonly used drugs that may have negative effect on glycemic control in patients with diabetes [13]. J. Zhou et al. in their article entitled "Effects of Simvastatin on Glucose Metabolism in Mouse MIN6 Cells" investigated the effects of simvastatin on insulin secretion in mouse MIN6 cells. Based on their findings, the authors proposed that simvastatin might inhibit insulin synthesis and secretion, thereby impairing glucose metabolism. These results are of paramount importance, given the widespread use of statins as drugs preventing cardiovascular disease.

Diabetes Self-Management Education (DSME) interventions have proven to help T2DM patients achieve and maintain optimal glycemic control [14]. The aim of the study "The Effect of Diabetes Self-Management Education on Body Weight, Glycemic Control, and Other Metabolic Markers in Patients with Type 2 Diabetes Mellitus" by C. Yuan et al. was to evaluate the effect of a short-term DSME on metabolic markers and atherosclerotic parameters in patients with T2DM. This work adds to the growing body of literature on the benefits that DSME can provide in diabetic patients.

Postprandial dyslipidemia is a common disorder in diabetes and it has been recognized as an important risk factor for CVD [15]. In their work entitled "Study of Postprandial Lipaemia in Type 2 Diabetes Mellitus: Exenatide versus Liraglutide," M. Voukali et al. investigated the effect of two GLP-1 receptor agonists, exenatide and liraglutide, on postprandial dyslipidemia. Both GLP-1 receptor agonists were equally effective in reducing postprandial lipemia. These findings give a more complete picture on the multiple mechanisms of action of GLP-1 receptor agonists.

Epidemiologic studies have shown an increased cancer incidence in diabetic patients, but the underlying mechanisms remain obscure [16]. The paper entitled "Real Life Cancer Comorbidity in Greek Patients with Diabetes Mellitus Followed Up at a Single Diabetes Center: An Unappreciated New Diabetes Complication" by A. Thanopoulou et al. aimed to determine cancer comorbidity in Greek patients with both diabetes types and to investigate the related risk factors. The authors suggest that we should develop strategies for early detection and prevention of certain types of cancer in diabetes.

Conclusions. All research and review papers included in this special issue highlight the fact that enquiry into diabetic complications is progressing. Although the papers published in the issue represent a small sample of the research in the field, they underline the complexity of the various etiological mechanisms and they illustrate the need of a comprehensive approach for the early detection and efficacious management of these chronic complications. Thus, we hope that the readers will find the content in this special issue to be a valuable resource for a better understanding of these important issues. This progress made in the field of diabetes research notwithstanding, we still have a long way to go until we succeed in unraveling the pathogenic mechanisms underlying these complications and in finding definitive cures.

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Maciej Banach
Michael Edmonds
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Research Article

Combined Methods for Diabetic Retinopathy Screening, Using Retina Photographs and Tear Fluid Proteomics Biomarkers

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Background. It is estimated that 347 million people suffer from diabetes mellitus (DM), and almost 5 million are blind due to diabetic retinopathy (DR). The progression of DR can be slowed down with early diagnosis and treatment. Therefore our aim was to develop a novel automated method for DR screening. *Methods*. 52 patients with diabetes mellitus were enrolled into the project. Of all patients, 39 had signs of DR. Digital retina images and tear fluid samples were taken from each eye. The results from the tear fluid proteomics analysis and from digital microaneurysm (MA) detection on fundus images were used as the input of a machine learning system. *Results*. MA detection method alone resulted in 0.84 sensitivity and 0.81 specificity. Using the proteomics data for analysis 0.87 sensitivity and 0.68 specificity values were achieved. The combined data analysis integrated the features of the proteomics data along with the number of detected MAs in the associated image and achieved sensitivity/specificity values of 0.93/0.78. *Conclusions*. As the two different types of data represent independent and complementary information on the outcome, the combined model resulted in a reliable screening method that is comparable to the requirements of DR screening programs applied in clinical routine.

1. Introduction

It is estimated that today more than 347 million people suffer from diabetes mellitus (DM) globally [1]. Diabetic retinopathy (DR), one of the most common complications of DM, accounts for about 5% of world blindness; this represents almost 5 million people in 2002. Approximately 80% of all patients with DM duration of at least 10 years suffer from some degree of DR [2]. During the development of DR, patients may not notice changes of their vision and DR might be very advanced by the time patients have visual complaints and experience visual loss eventually.

In order to detect DR in an early stage everyone with DM should be subjected to comprehensive dilated eye exam at least once a year [3]. In case of early diagnosis, the progression of DR can be slowed down by appropriate systemic or local therapy.

More than two-thirds of patients with DM live in developing countries such as India (32 million) and China (21 million) where the access to high quality health care system

is not universal [4]. As a result of the high human resource cost of the DR screening programs, developed countries are also looking for more cost-effective and scalable alternatives to alter the existing methods.

Involvement of human graders is currently universal and the less automation is in the system the greater the costs are. Where possible, the digital photography method is the screening method of choice [5], although many countries rely on traditional clinical examination.

Recent photographic methods employ digital images evaluated by qualified personnel. Based on mydriatic 45° retinal photographs examined by trained staff, the sensitivity for the detection of sight-threatening DR has been reported to range between 87 and 100% with specificities of 83–96% [6]. The expected values for DR screening program as specified by the British Diabetic Association (Diabetes UK) are at least 80% sensitivity and 95% specificity [7, 8].

Quality control concerns and cost efficiency have brought forward the need for regular and centralized DR screening in several developed countries [9]. Digital images are captured at healthcare facilities and evaluated at grading centers by qualified personnel or ophthalmologists [10]. The process performs with high accuracy; however, it may not be scalable and sustainable in economically challenged countries as it requires intensive technical competence [11].

To improve the cost-effectiveness of DR screening several image processing based methods have been developed in the last decade providing an alternative to first phase examinations performed by human graders [12, 13]. In this case, the role of human graders would be reduced to examine true positive or ambiguous images as well as perform quality control on images that were described as normal by the software after the automated prescreening process [14].

The hallmark of DR is microaneurysms (MAs). As these outpouches occur on small blood vessels, most of the image processing based screening methods concentrate on the detection of this type of lesion. The International Retinopathy Online Challenge offers an opportunity to compare the results of image processing based algorithms for DR identification via MA detection, where a screening system developed by our research group achieved the best outcome in 2010 [15]. The specificity and sensitivity values of the automated MA detectors are close to those of human graders [16–18]. Despite the promising initial results, the use of image processing based methods is limited in the clinical routine, probably because their sensitivity and specificity cannot be improved further. Nevertheless automated image clarity assessment has been used in some places prior to human grading, for example, the Scottish Screening Programme [19].

In this study, in order to increase the sensitivity and specificity values of the photographic screening method, we used an MA detector combined with tear fluid proteomics based methodologies in one single system. The rationale behind this was to use two independent but complimentary techniques.

Currently tear fluid proteomics based methods are not used in the clinical routine either. Protein profile changes in tear fluid under abnormal pathological and physiological conditions, such as inflammatory diseases or wound healing, have been verified by several studies [20].

While vitreous humour proteome changes are known to be more specific [21], implementing vitreous humour proteomics in daily clinical practice is nevertheless problematic as it requires invasive method of sampling.

However, tear fluid sampling may be an efficiently standardizable noninvasive process. In our previous study, we analyzed the alteration of tear protein concentrations in DR in order to determine which proteins, as potential biomarkers, are found in tear fluid in DR patients [22].

In a previous paper our research group published the first attempt for using tear fluid proteomics multimarkers for DR screening. We applied machine learning algorithms to predict whether the given patients with DM suffer from DR or not, based on the global proteomics pattern changes of their tear fluid. In that study we concluded that, due to its low sensitivity (74%) and specificity (48%) values, the proteomics based screening method alone is not appropriate for clinical application at its present format. However, in combination with other methods, it is able to improve the performance of a combined system [23].

2. Materials and Methods

In the study we developed three automated screening systems. The first used image processing based algorithms in order to detect MAs in digital retina images. The second was based on the evaluation of tear fluid protein multimarkers. The third system combined the above mentioned MA detection and tear fluid proteomics analysis. Thereafter we assessed the performance of all three methods. Our aim was to develop an automated DR screening system that reaches the sensitivity and specificity values of screening performed by human graders. In this way, compared to our previous work, this new method represents significant improvement.

2.1. Patient Examination. Altogether, 52 patients with DM were recruited into the study (21 males; 65.2 years average age; 16.4 years average duration of diabetes; 14 NIDDM) from the Ophthalmology Outpatient Clinic of the University of Debrecen. At the time of the patient examination all the patients were under antidiabetic medication. Out of all patients, 39 had signs of DR. 16 patients out of the 39 have undergone one or more laser photocoagulation treatment. In our study, we only involved the eyes of the patients of which we could obtain complete tear fluid proteomics data and clinically evaluable fundus photos. Out of the potential 104 eyes, 74 had corresponding tear fluid analysis and gradable digital retinal images. Although the remaining 30 eyes were also examined clinically, either difficulties in tear fluid sampling, for example, noncompliance, operated eye, and keratoconjunctivitis (9 participants), or difficulties during the digital retina photography, for example, hemorrhage, angle-closure glaucoma, and cataract (21 participants) led to no procurement of relevant corresponding information as shown in Table 1.

Table 1: Characteristics of the participants.

Total number of participants enrolled			Eye examination		
52			104		
Non-DR	DR		Eyes included	Eyes excluded	
	Proliferative	Nonproliferative		Tear fluid sampling	Retina photography
13	15	24	74	9	21

DR was determined by first capturing and then grading standard 7-field fundus images. The images were taken by Megaplus Camera Model 1,6i/10 BIT Zeiss (Carl Zeiss Ophthalmic System A6, Jena, Germany). All of these were assessed by two independent ophthalmologists.

The collected tear fluid samples were obtained under standardized conditions by a qualified assistant [24]. The samples were acquired using glass capillaries directly before the pupil dilatation from the lower tear meniscus (a horizontal thickening of the precorneal tear film by the lower margin) at the lateral canthus and care was taken not to touch the conjunctiva. The time of the sampling procedure was noted. The secretion rate was calculated by dividing the collected tear volume by the time of sample collection and was given in μL/min. Samples used in this analysis had secretion rates of 5–15 μL/min.

2.2. Machine Learning. During the project data processing was performed by using machine learning algorithms. Machine learning is a subfield of artificial intelligence and it deals with the question as to how to construct computer programs that can learn from data. The core task of machine learning is performing inference from new samples. In supervised learning the goal is to predict the value of an outcome measure based on a number of input measures. Supervised learning takes a known set of input data (training set) and known responses (labeled output) to the data and seeks to build a predictor model that generates reasonable predictions for the response to new data.

In our case, the input data come from proteomics experiments and from image processing based method, while the predicted outcomes concern whether the certain patient has DR or not. The model makes predictions using new data to classify patients according to their tear proteomics data and digital retinal image gradings and forms a well-defined way to allow prescreening activities.

In the scientific literature no report can be found about the possibility and potential relevance of combining tear fluid proteomics and image processing on DR screening; the method proposed by this paper is unprecedented.

2.3. Microaneurysm Detector. The image data was processed using an MA detector, based on the image processing techniques (Figure 1) [25, 26]. In short, the green plane of the image is shade corrected, by subtracting the median filtered version of the image (using a 35 × 35 rectangle) from the green component of each pixel value. On the shade corrected image, a contrast limited histogram equalization (CLAHE) is performed, which is used to enhance the contrast of the image.

This was followed by a 3 × 3 median filtering for smoothing. The next step of the processing is a top hat transform by morphological reconstruction [27]. Top hat transform is an image processing method used for small feature extraction.

The reconstructed image is opened by a 10×10 disk shape to detect only small circular objects, resulting in candidate MAs. The center of the candidates is calculated and the following features are extracted for each candidate: area, rotational inertia, mean intensity in the morphologically opened image, mean intensity in green plane, and standard deviation of intensity in green plane.

The Gradient Boosting Machine (GBM) model was used to classify candidates MAs based on the above features [28, 29].

The testing was done based on hand marked MAs of the retina images.

2.4. Tear Fluid Proteomics Based Method. Immediately after sampling, the samples were centrifuged (1800 rpm) for 8–10 minutes and supernatants were deep-frozen at −80°C and were thawed only once for measurements.

Protein identification was done from the tear fluid of each of the patients. Tear samples were examined using nano-HPLC coupled ESI-MS/MS mass spectrometry protein identification as described elsewhere [22, 30, 31].

2.5. Application of Machine Learning Methods. Machine learning method provided the basis of our DR screening procedure. We have used learning data sets in order to teach the machine learning algorithms to predict the occurrence of certain future events, using empirical data containing incomplete information. In our case the learning datasets were the proteomics profiles, the retinal images, and the clinical diagnosis (DR/non DR) of the enrolled diabetes patients with DM.

Three classifiers were trained on the datasets; the first model was based on fundus image data alone, the second on using proteomics data, and the final was the combined model. During the process we used the best performing machine learning algorithms, Naive Bayes Classifier in the first and GBM in the other two models. 10-fold cross-validation (repeated 10 times) was used to assess the performance of this classifier.

The fundus images were processed using the MA detector algorithm, and a threshold was chosen for a candidate to be classified as MA. The count of MAs on an image found this

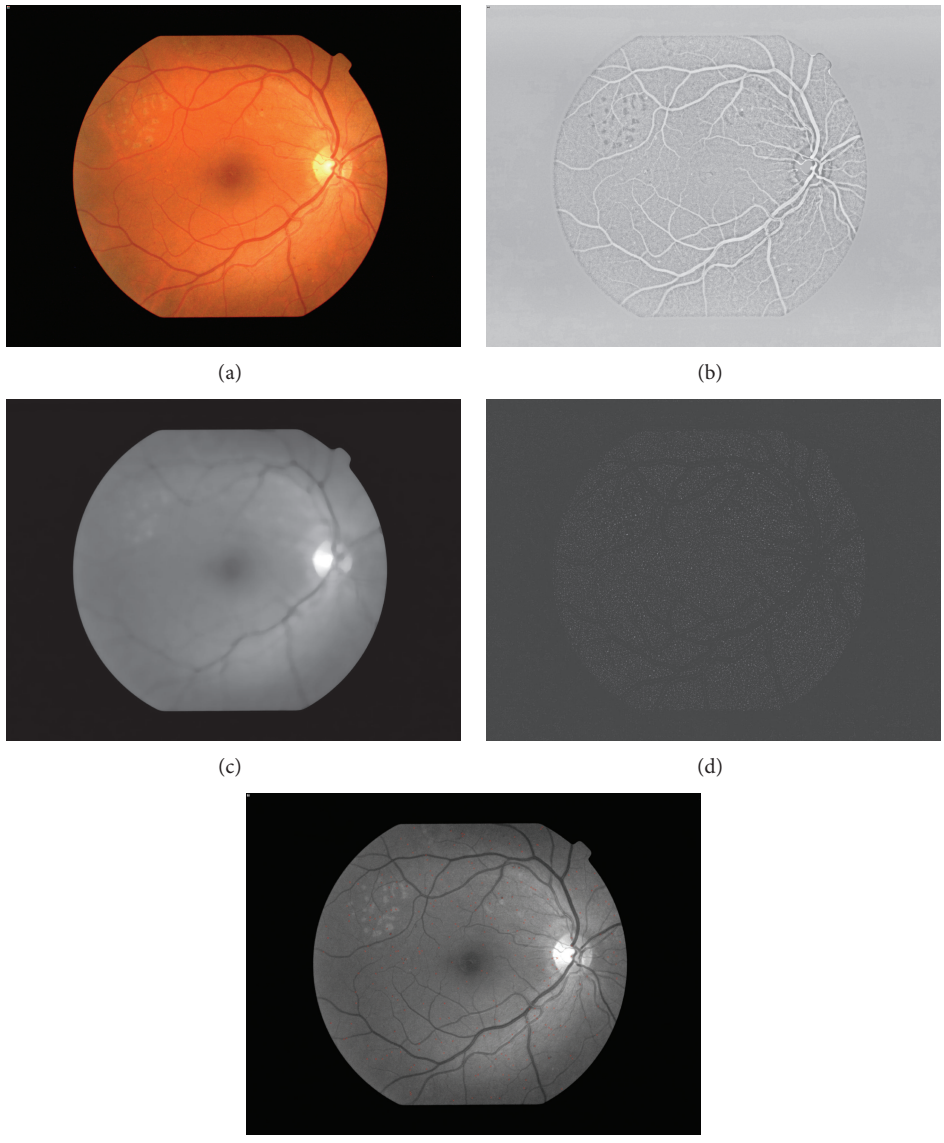

Figure 1: Microaneurysm detection. (a) Original retina image; (b) CLAHE contrast enhancement; (c) median filtering; (d) top-hat transform; (e) raw MA candidates.

way was used as the only feature for detecting DR, in case of the first model. In Table 2, our results are reported at image level.

We have used naive Bayes classifier for this analysis.

In the second model the global pattern of protein concentrations and its changes were described by the examination of the concentrations of 34 different proteins.

For the combined data analysis our input data were protein levels measured in tear fluid samples from patients with diabetes, the number of detected MAs in the associated images, and clinical data regarding their DR status.

As a next step, following the learning process, we intended to assess the performance of the screening method. At this phase only the protein levels and retinal images were entered into the system without diagnosis. Our goal was to show that the model based on both types of data has better performance than the models based on either the image data or the proteomics data alone. Figure 2 shows the learning and the assessment phases of the application of the machine learning algorithm.

We used k-fold cross-validation method to evaluate the classifier's performance based on the three dataset setup. In k-fold cross-validation the data is first partitioned into k equally (or nearly equally) sized segments or folds [32].

Afterwards k iterations of training and validation are performed. Within each iteration different subsets of the data are kept for validation process while the remaining $k - 1$-fold are used for learning. At the end of the cross-validation, the

Table 2: Performance measures of the screening methods.

Screening method		SENS	SPC	ACC	PREC	NPV	$F1$	LRP	LRN
Image processing	Mean	0.84	0.81	0.84	0.94	0.63	0.89	4.42	0.20
	SD	0.11	0.04	0.10	0.13	0.11	0.11	2.36	0.11
Proteomics	Mean	0.87	0.68	0.82	0.89	0.63	0.88	2.72	0.19
	SD	0.17	0.12	0.11	0.21	0.15	0.16	1.24	0.12
Combined method	Mean	0.93	0.78	0.89	0.93	0.78	0.93	4.23	0.09
	SD	0.18	0.19	0.15	0.23	0.22	0.18	1.32	0.07

Performance measures of the image processing based, the tear proteomics based, and the combined screening methods. SENS: sensitivity, SPC: specificity, ACC: accuracy, PREC: precision (positive predictive value), NPV: negative predictive value, $F1$: F-measure, LRP: likelihood ratio positive, and LRN: likelihood ratio negative.

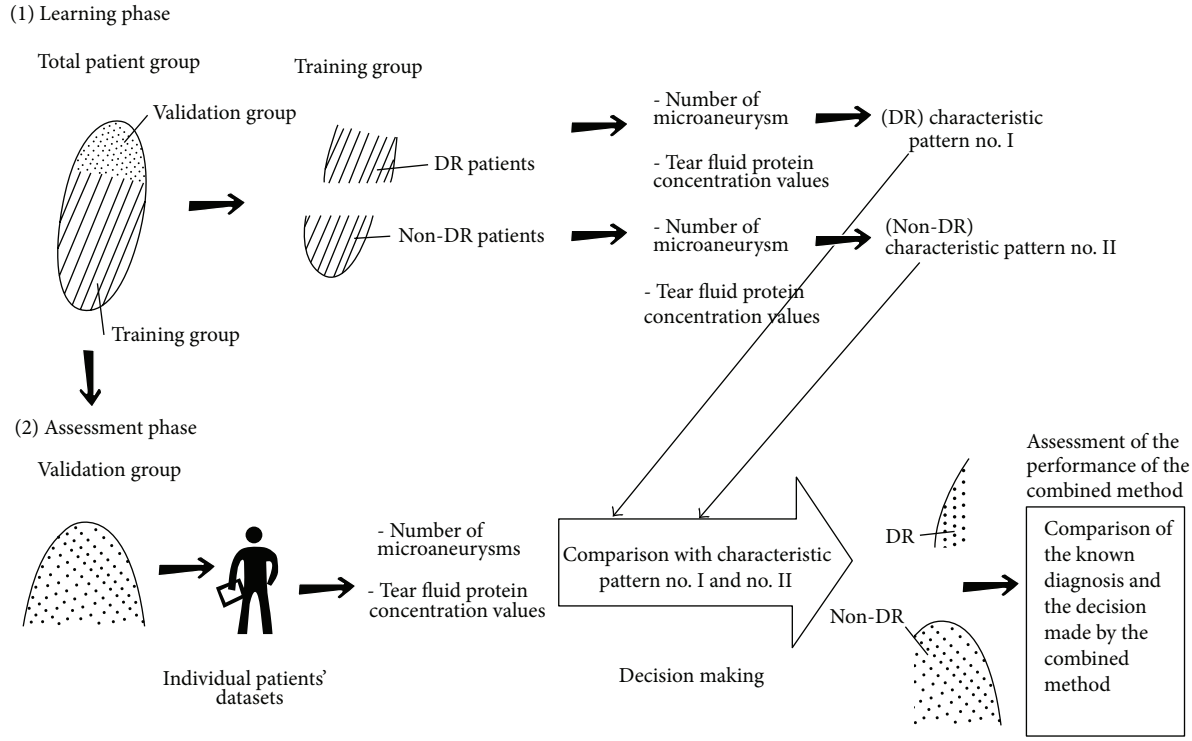

Figure 2: Application of machine learning algorithm in the combined model. Learning phase (above): we randomly select a subpopulation of the total patient group, called the training group, and then use the known clinical diagnosis to split the training group into a DR group and a non-DR group. The clinical diagnosis, the number of MAs on the retina images, and the protein concentration values are the inputs of the machine learning algorithm. The algorithms are able to tell which data patterns are the most characteristic for the DR and non-DR groups. Assessment phase (below): in the following steps, we use the data from the validation group. The number of MAs and the protein concentration values constitute the input of the algorithm, but we do not use the information from clinical diagnosis. The learning algorithm compares the new data to the characteristic patterns that are known from the learning phase and will make its own decision (normal/DR) for each patient as the output of the model. For the assessment of the performance of the model, we compare the output with the known clinical diagnosis.

estimate is determined as the mean of the features of the k-fold model where we used 10-fold cross-validation, repeated 10 times.

With this basic form of cross-validation we obtained estimations for many performance indicators such as specificity, sensitivity, accuracy, and F-measure (harmonic mean of precision and sensitivity as a single measure for the performance of the model).

During the 10-fold cross-validation process, the content of learning and validation datasets has been randomly chosen. In certain cases, one eye of a person was assigned to the validation and the other to the learning dataset. Considering the relatively low number of participants enrolled in the project, this setting may increase significantly the performance of the image processing based method. Therefore, we tried to exclude this potential bias from our experiments.

When the software splits the total patient group into a validation and training set, we exclude the eyes from the training set whose pair was in the validation set.

2.6. Data Analysis: Software Tools. We used the Smartbiobank data management system for the collection of experimental (proteomics) and clinical data (DR/Non-DR) with the related retinal images [33]. During the data analysis process the *R* statistical framework and the following packages have been used: "gbm", "caret", "stat", and "e1071" [34]. The image processing tasks were done using the Octave software [35].

3. Results and Discussion

In case of the first model MA-count was used as the only feature for detecting DR. MA detection method alone resulted in 0.84 sensitivity and 0.81 specificity values.

Using the proteomics data for analysis 0.87 sensitivity and 0.68 specificity values were achieved by using GBM models.

The combined model resulted in a more powerful classifier, achieving 0.93 sensitivity and 0.78 specificity values, as the two different types of data provide independent and complementary aspects of the underlying information of the outcome.

Data in Table 2 demonstrate that the MA detector alone performs significantly better than the tear proteomics based method. Regarding accuracy measurement, the combined method exceeds both image processing and proteomics based methods values. Specificity value of the MA detector slightly outperforms the specificity of the combined method as well. Nevertheless, taking everything into consideration, the use of proteomics data definitely improves the performance of the MA detector; therefore the combined screening method is the best classifier out of the three.

Over the last decade, several studies have been published on the application of image processing methods for DR screening. These methods, despite their promising performances, are only slowly being applied in clinical practice.

In parallel, our team published the first attempt of using tear fluid proteomics based method for DR screening. Although the conclusion of this work was that this method alone is not accurate enough for clinical use [23], there is a definite scope for the improvement of the performance of proteomics based classifier. With improvements in tear fluid analysis, this method might become clinically useful with time, as it requires little equipment for obtaining the sample making potential mass-screening of DR in hard-to-reach areas viable.

In theory, the performance of a classifier might be improved by using the combination of different predictor models [36, 37]. Combined diagnostic approaches are also used in the routine medical practice to improve the clinical effectiveness of existing screening programs [38].

Although there are numerous screening methods published to be comparable to human graders, there is a lack of systems that have been validated on independent cohort using internationally recognized DR standard. Abramoff and his workgroup published a method using combined MA, hemorrhage, cotton wool spots, and exudates detection on a diabetes at risk cohort of 874 participants. They completed the method with the detection of irregular lesions such as large hemorrhages and neovascularization. The sensitivity of the system was 96.8% (95% CI, 94.4%–99.3%) with 59.4% (95% CI, 55.7%–63.0%) specificity. The relatively low sensitivity value limits the usability of the system [39].

The automated grading method published by Goatman and his colleagues intended to remove normal images from the image database before manual grading, thus reducing the manual workload. The combined method was based on the detection of MA, blot hemorrhages, and exudates. According to the well-known tradeoff between sensitivity and specificity, the 100% sensitivity is coupled with low specificity value.

Considering that the system is proposed to be a prescreening tool, the workload reduction, ranged from 26.4% (MA/BH/EX, both fields) to 38.1% (MA only, macular field), is remarkable [40].

As we mentioned earlier, tear fluid proteomics based methods are not used in the clinical routine. In experimental settings the tear fluid proteomics based screening methods, because of the expensive MS/MS experiments, limit the application of larger sample sizes that are used in image processing based projects.

Protein biomarkers and the MAs on retinal images represent different data sources and information on DR eye. Our results of 0.93 sensitivity and 0.78 specificity values are close to reach the threshold recommended for routine clinical screening of 80% and 95%, respectively. Considering the 9 eyes for tear fluid proteomics examination and the 21 eyes for which the image processing cannot be performed, the clinical examination protocol should be improved in the future. Our future aim is to decrease the number of the protein biomarkers applied in the screening system in order to replace the MS/MS method to a more cost-effective rapid test. To keep the performance of the method we use Principal Component Analysis (PCA) to select the potential biomarkers with the highest predictive values. For this lower number of protein marker we intend to define a concentration threshold instead of using the absolute values of the concentrations. With the achievement of this objective we will be able to develop a rapid test that can be performed together with the retina photography. Potentially the protein rapid test can be performed at the diabetes patient's home using a kit provided by the family practitioner or the screening center via mail.

4. Conclusion

Our findings suggest that the maximal performance of this method has not been reached yet. Results can be improved in three potential ways: (i) by optimizing the parameter settings of both tear fluid proteomics and MA detector based classifiers; (ii) by comparing and choosing the best classifier available for the combined screening method; (iii) by fine tuning the patient examination and lab diagnostic protocols.

Considering that we intend to develop a prescreening method, in order to decrease the workload of the DR screening centers, with the maximization of our system sensitivity,

in a subsequent study, the potential workload reduction also could be assessed.

Both tear sampling and retina photography are noninvasive methods and can be implemented at general practitioner's settings. For the assessment of cost-effectiveness of the method further analysis is needed. However, it is expected in the future that the cost of human resources in clinical care becomes higher, in parallel with a rapid decrease in the cost of IT and laboratory technologies. In light of these changes the combined method of tear fluid proteomics and computer-assisted image processing of digital retinal photographs may provide a promising alternative in DR screening.

Ethical Approval

Ethical approval number is IX-R-052/00016-26/2012. The study has conducted in accordance with the Declaration of Helsinki (1964).

Consent

We certify that during this research we followed all the applicable institutional and governmental regulations concerning the ethical use of human volunteers and the study was performed with informed consent.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Protective Effects of the Mushroom *Lactarius deterrimus* Extract on Systemic Oxidative Stress and Pancreatic Islets in Streptozotocin-Induced Diabetic Rats

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The aim of this study was to assess the *in vivo* effects of the extract of the medicinal mushroom, *Lactarius deterrimus*, when administered (60 mg/kg, i.p.) daily for four weeks to streptozotocin- (STZ-) induced diabetic rats. Diabetic rats treated with the *L. deterrimus* extract displayed several improved biochemical parameters in the circulation: reduced hyperglycemia, lower triglyceride concentration and reduced glycated hemoglobin, glycated serum protein, and advanced glycation end product (AGE) levels. This treatment also adjusted the diabetes-induced redox imbalance. Thus, higher activities of the antioxidative enzymes, superoxide dismutase, and catalase in the circulation were accompanied by increased levels of free intracellular thiols and glutathionylated proteins after treatment with the *L. deterrimus* extract. In addition to a systemic antioxidant effect, the administration of the extract to diabetic rats also had a positive localized effect on pancreatic islets where it decreased AGE formation, and increased the expression of chemokine CXCL12 protein that mediates the restoration of β-cell population through the activation of the serine/threonine-specific Akt protein kinase prosurvival pathway. As a result, the numbers of proliferating cell nuclear antigen- (PCNA-) and insulin-positive β-cells were increased. These results show that the ability of the *L. deterrimus* extract to alleviate oxidative stress and increase β-cell mass represents a therapeutic potential for diabetes management.

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder caused by absolute insulin deficiency or insufficient insulin secretion and/or insulin sensitivity [1] and is characterized by hyperglycemia. Pancreatic β-cells are responsible for insulin production and maintenance of blood glucose concentrations [2]. β-cell dysfunction and declining β-cell numbers are responsible for the loss of endocrine pancreas function in both type 1 (T1D) and type 2 diabetes, albeit loss of β-cells is more rapid in autoimmune destruction in T1D. Hyperglycemia is at the root of the significant increase in the formation of toxic reactive oxygen species (ROS) and establishment of oxidative stress that is responsible for the progression of diabetes and its complications [1], with vascular injury, hypertension, nephropathy, retinopathy, and neuropathy as major outcomes.

Despite many strategies and agents, DM management requires constant improvement. Considering that synthetic drugs have specific limitations in treating diabetic complications, it is important to refine approaches based on novel natural compounds that could support the restoration and maintenance of pancreatic β-cell numbers and assist in controlling diabetic complications [3–5]. Since oxidative stress is an

essential contributor to the development and progression of diabetes and related complications, the therapeutic approach to DM is based on improving hyperglycemia and the organism's endogenous antioxidant activities. Recent investigations that have provided evidence for the antidiabetic effectiveness of mushrooms provide novel approaches for controlling DM and its complications [6]. Edible mushrooms are rich in vitamins (B, D, A, C, K), contain high protein contents and minerals, and are low in saturated fats. Many of the biological characteristics of mushrooms are mainly due to the presence of dietary fiber, in particular chitin and the α and β glucans that have significant nonspecific immunostimulatory effects [7, 8]. Mushrooms have also proven to be effective ROS scavengers, their antioxidant properties correlating with their total content of phenolics [4, 9, 10].

Lactarius deterrimus, also known as false saffron milkcap, is an edible mushroom from the family Russulaceae that mainly grows in coniferous woods in northern, northeastern, and Central Europe. *L. deterrimus* and other *Lactarius* species, such as *Lactarius salmonicolor*, *Lactarius deliciosus*, and *Lactarius sanguifluus*, possess potent medicinal activities. The antimicrobial activities against several Gram (+) and Gram (−) bacteria and the anticancer and antiviral activities of their antioxidant constituents have been described; in addition, it has been suggested that these mushrooms represent a potential source of natural immunostimulatory substances [11–13]. Previously, we described the *in vitro* antioxidant and scavenging properties of the *L. deterrimus* extract, especially NO-scavenging and metal-chelating activities, which correlated with the ability of the extract to prevent lipid peroxidation and DNA damage during streptozotocin-induced oxidative stress in Rin-5F cells [14].

The aim of this study was to examine the potential *in vivo* beneficial effect of the extract obtained from the edible mushroom *Lactarius deterrimus* on the systemic antioxidant status and control of pancreatic damage in streptozotocin-(STZ-) induced diabetic rats.

2. Materials and Methods

2.1. Mushroom Collection and Extract Preparation. The mushroom *Lactarius deterrimus* was collected near the village Mune from the Istra region in Croatia in the summer of 2008. Fruiting bodies were gently cleansed of any residual compost. Fresh mushrooms were air-dried and stored in airtight plastic bags at room temperature. The dried mushroom samples were broken up in a blender before extraction with 50% ethanol at a sample : solvent ratio of 1 : 10 (w/v). The extraction process was carried out using an ultrasonic bath (B-220; Branson and SmithKline Company) at 45°C for 40 min. After filtration, the extraction solvent was removed by a rotary evaporator (Devarot; Elektromedicina) under vacuum. The obtained *L. deterrimus* extracts (Ld) were dried at 60°C to a constant mass and stored in glass bottles at −80°C to prevent oxidative damage.

2.2. Phytochemical Analysis of the L. deterrimus Extract. The total phenolic compounds and other oxidation substrates contained in dry mushroom extracts were determined by the Folin-Ciocalteu colorimetric method based on absorbance at 765 nm [15] and are shown in Table 1. The values are expressed as g of gallic acid equivalents (GAE) per 100 g of the dry mushroom extract sample. The total flavonoid content was analyzed by the aluminum-chloride colorimetric assay at 510 nm [16] and is expressed as g of quercetin equivalents (QE) per 100 g of the dry extract sample. Previous detailed qualitative and quantitative analyses of the extract by high-performance liquid chromatography with diode-array detection (HPLC/DAD) revealed the presence of tryptophan, p-hydroxybenzoic acid, and unsaturated oxy(hydroxyl- or epoxy-) fatty acids [14].

Table 1: Phytochemical constituents of the *L. deterrimus* extract.

	Phenolic content (mg GAE/g extract)[a]	Flavonoid content (mg QE/g extract)[b]
Lactarius deterrimus	14.8 ± 2.23	5.07 ± 1.97

Total phenolic and flavonoid contents were determined by the Folin Ciocalteu and aluminium-chloride colorimetric methods, respectively, and are expressed as milligrams of gallic acid (GA) per gram of dry mushroom material[a] and as milligrams of quercetin (QE) per gram of dry mushroom material[b]. Data are presented as means ± SD.

2.3. Animals. Experiments were performed on 2.5-month-old adult albino Wistar rats weighing 220–250 g. All animal procedures were in compliance with the EEC Directive (86/609/EEC) on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade.

2.4. Experimental Design. The experimental model of multiple low dose (MLDS) STZ-induced diabetes was used. Diabetes was induced by injection (i.p.) of STZ (40 mg/kg/day) to Wistar rats for five consecutive days. STZ was dissolved before use in sodium citrate buffer (0.1 M, pH 4.5). Blood glucose was measured 24 h after the last STZ injection. Blood samples were obtained from the tail vein of rats that fasted overnight, and glucose was measured with a blood glucose meter (Accu-Chech Active). Rats were considered to have diabetes when the fasting blood glucose level exceeded 20 mmol/L (the baseline glucose level measured before diabetes induction by STZ was 6.2 ± 0.3). Male albino Wistar rats were randomly divided into four groups: (i) NDM: the nondiabetic group ($n = 7$), also referred to as the negative control, received citrate buffer (i.p.) equivalent to the STZ injection for 5 consecutive days; (ii) NDM + Ld: the nondiabetic group ($n = 7$), also referred to as the positive control, was administered the mushroom extract (Ld; 60 mg/kg, i.p.) daily for four weeks; (iii) DM: the diabetic group ($n = 8$) was injected STZ and left untreated throughout the four week period; (iv) DM + Ld: the diabetic group ($n = 8$) was treated with the Ld daily for four weeks, starting from the last day of STZ administration.

2.5. Serum and Hemolysate Preparation. Rat blood serum was collected after blood clotting and centrifugation at 2000 ×g for 10 min. The serum was used for determination of glycated proteins and the amounts of protein sulfhydryl groups (–SH). For the preparation of red blood cell (RBC) hemolysates, blood was collected in heparinized tubes (1 000 IU heparin) and centrifuged at 2000 ×g for 10 min. RBCs were washed twice with 0.9% NaCl and centrifuged. The washed RBCs were lysed in 3 volumes of cold water for 30 min on ice. Hemolysates were used for the determination of catalase (CAT), superoxide dismutase (SOD), glutathionylated proteins (GSSP), hemoglobin (Hb), and glycated (Gly) Hb.

2.6. Determination of Biochemical Parameters of Diabetes. Blood glucose levels were measured with a commercial kit (Gluco-quant Glucose/HK, Boehringer Mannheim, Germany) based on the hexokinase/G6P-DH enzymatic method. GlyHb was determined by the colorimetric assay according to Parker et al. [17]. Serum triglycerides were measured by the glycerol-3-phosphate (GPO) oxidase-p-aminophenazone (PAP) method with an enzymatic kit (Randox Laboratories, UK). Detection of glycated serum proteins was by the fructosamine assay. The glycation of serum proteins was measured according to Johnson et al. [18]. Aliquots of sera (50 μL) were added to 450 μL of 100 mM carbonate buffer (pH 10.8) containing 0.5 mM nitro blue tetrazolium (NBT). The samples were incubated for 1 h at 37°C. Absorbance was measured at 595 nm.

2.7. Determination of GSSP in the Circulation. Acid-precipitated proteins in hemolysates were thoroughly washed with the precipitating solution until no trace of soluble reduced glutathione (GSH) or glutathione disulfide (GSSG) was detected. The pellets were then resuspended and brought to an alkaline pH (pH 7.5–8) for 5–30 min. Under these conditions, GSH is released via an –SH/disulfide (–S–S–) exchange reaction. The reaction was stopped by the addition of trichloroacetic acid to a final 5% concentration. The amount of released GSH was determined enzymatically in the supernatants after centrifugation.

2.8. Determination of Protein Sulfhydryl Groups in Circulation. Protein –SH groups were determined by Ellman's method [19]. Briefly, 0.5 mL of serum was added to a cuvette containing 0.1 M phosphate buffer, pH 7.4 (0.5 mL); 0.2 mL of 3 mM 5,5′-dithiobis (2-nitrobenzoic acid) was then added to start the reaction. Absorbance was measured at 412 nm after 10 min. The number of –SH groups was calculated according to the following formula:

$$\text{mol –SH/g proteins} = \left[\frac{A \text{ sample}}{14150} \times \text{dilution factor}\right] \text{/g proteins.} \quad (1)$$

2.9. Determination of SOD and CAT Activities in the Circulation. CAT activity was determined according to Beutler [20] as the rate of hydrogen peroxide decomposition and expressed as μmol H_2O_2/min/g Hb. The Hb was removed from hemolysates prior to measurement of SOD activity by the epinephrine method [21]. One unit of SOD activity was defined as the amount of Hb that causes 50% inhibition of adrenaline autoxidation.

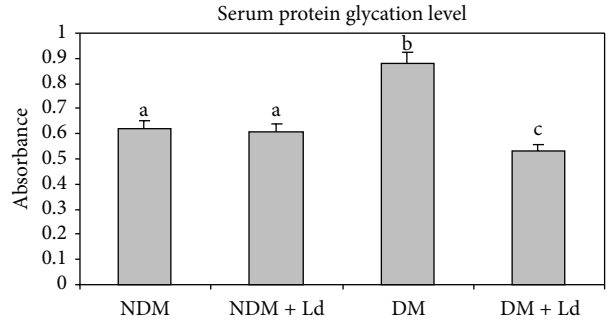
2.10. Determination of AGE in Circulation. The fluorescent products of AGE were detected according to Munch et al. [22], Henle et al. [23], and Kalousová et al. [24]. Blood serum was diluted 1 : 50 with phosphate buffered saline (PBS) pH 7.4 and fluorescence intensity was measured at an excitation wavelength of 350 nm and an emission wavelength of 440 nm with a luminescence spectrometer (LS50B; Perkin-Elmer Ltd., Buckinghamshire, England). The fluorescence intensity was expressed in arbitrary units.

2.11. Histological Analysis and Immunostaining. The pancreata from all experimental groups were removed and fixed in 10% buffered formalin for histological and immunohistological examination. Pancreatic tissues from all examined groups were blocked in paraffin and sectioned at 5 μm thickness for histological and immunohistochemical examination (Leica DMLB; objective magnification 40x). For histological analysis tissue sections were stained with hematoxylin and eosin and observed under a light microscope. For immunohistochemical analysis, deparaffinized sections were passed through xylene and rehydrated in sequentially graduated ethyl alcohol. Slides were incubated in 0.3% hydrogen peroxide/methanol for 20 min to reduce nonspecific background staining due to endogenous peroxidase. After washing in PBS, the sections were treated with 0.01 M sodium citrate buffer at 98°C for antigen retrieval. The cooled tissues were washed four times in PBS prior to application of the blocking serum for 5 min (0.05% Tween 20, 5% bovine serum albumin). The primary antibody was applied overnight at room temperature (RT). Polyclonal antibodies raised against insulin, CXCL12, C-X-C chemokine receptor type 4 (CXCR4), N(ε)-(carboxymethyl)lysine (CML), an advanced glycation end product (AGE), receptor for AGE (RAGE), phosphorylated protein kinase B (pAkt), and proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted 1 : 50 in PBS with 2% dry skimmed milk. After washing in PBS, sections were incubated at RT for 1 h with secondary antibody horseradish peroxidase (1 : 100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Tissues were incubated for 20 min at RT in a solution of 3,3′-diaminobenzidine (DAB). After washing with PBS, the tissues were counterstained with haematoxylin and washed in water, and the coverslips were applied with mounting media. For the negative control, the primary antibody was not added to the sections.

2.12. Statistical Analysis. The data were expressed as the mean ± S.E.M. (standard error of mean). Statistical differences between groups were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test.

	NDM	NDM + Ld	DM	DM + Ld
Glucose (mmol/L)	6.2 ± 0.2^{a}	6.5 ± 0.3^{a}	30.1 ± 1.1^{b}	22.7 ± 0.9^{c}
Total glycated Hb (μmol fructose/gHb)	6.5 ± 0.3^{a}	6.7 ± 0.3^{a}	11.6 ± 0.5^{b}	9.1 ± 0.4^{c}
Triglycerides (mmol/L)	0.75 ± 0.03^{a}	0.5 ± 0.02^{a}	1.4 ± 0.06^{b}	1 ± 0.03^{c}

(a)



(b)

Figure 1: The effect of *L. deterrimus* extract administration on the biochemical parameters in sera (a) and glycation levels of serum proteins (b). NDM: control rats; NDM + Ld: control rats treated daily with *L. deterrimus* extract for four weeks; DM: STZ-induced diabetic rats; DM + Ld: STZ-induced diabetic rats treated with *L. deterrimus* extract for four weeks. Hb: haemoglobin. The values are presented as the mean ± S.E.M.; values not sharing a common superscript letter differ significantly at $P < 0.05$.

The difference was considered statistically significant at $P < 0.05$.

3. Results

3.1. Biochemical Parameters of Diabetes in Rats Treated with the L. deterrimus Extract. Administration of Ld led to an overall improvement of the main biochemical parameters of diabetes, albeit short of their restoration to their respective physiological levels. As can be seen in Figure 1(a), treatment of diabetic animals with Ld reduced the glucose concentration by almost 25% and lowered the level of triglycerides by about 28% and GlyHb by about 21%. The treatment also lowered the level of serum protein nonenzymatic glycation to the level measured in control rats (Figure 1(b)). Ld-treated nondiabetic rats did not exhibit any changes of these parameters when compared to the control group.

3.2. Oxidative Stress and Antioxidative Protection in the Circulation of Rats Treated with the L. deterrimus Extract. The redox status in experimental rats was estimated by measuring the levels of free intracellular thiols (Figure 2(a)) and GSSP (Figure 2(b)) that are directly linked to the redox state of the cell [25, 26]. These experiments revealed that the treatment with Ld significantly improved the redox parameters in the circulation. While the level of serum protein –SH groups was significantly decreased in diabetic rats, the treatment with Ld restored the –SH content almost to the control level. The –SH content was not significantly changed when control rats were administered the extract. As can be observed in Figure 2(b), the 1.3-fold increase in the level of GSSP in diabetic rats as compared to the control level was brought to the control level by the administration of Ld. The GSSP level was not significantly changed when healthy rats were administered Ld.

The activities of the major antioxidant enzymes, SOD and CAT, provide the first line of antioxidant defenses and protect cells from ROS damage [27]. As can be seen in Figure 2(c), the decreased levels of SOD and CAT activities in the circulation of diabetic rats were improved after Ld administration, the effect of Ld being more pronounced on CAT activity. In addition, a significant increase in the SOD/CAT ratio was observed under diabetic conditions, while the Ld treatment reduced this ratio to the control level in diabetic animals. SOD and CAT activities did not change after Ld administration to control rats.

3.3. L. deterrimus Extract Administration Induces Histological Changes and Stimulates the Production of Insulin and CXCL12 in Pancreatic Islets. Hematoxylin and eosin sections (Figure 3) show that the pancreas of control rats is comprised of numerous, compactly arranged cells in the islets of Langerhans that appear as dense cords. The pancreas of diabetic rats had smaller pancreatic islets with lower numbers of β-cells, displaying increased vacuolation and clumped cells. Ld-treated diabetic rats resembled more closely normal islet cell architecture, which is suggestive of a protective role of Ld on the pancreas of diabetic rats (Figure 3, HE). Immunohistochemical staining with insulin revealed disorganized islets in diabetic rats, with unevenly distributed insulin-positive cells in comparison to control islets that displayed strong insulin immunostaining (Figure 3, insulin). The Ld treatment increased the number of insulin-positive cells in the islets of diabetic rats (Figure 3, insulin).

The chemokine CXCL12 and its receptor, CXCR4, have been shown to mediate β-cell repair [28]. Immunohistochemical staining revealed the presence of CXCL12-positive

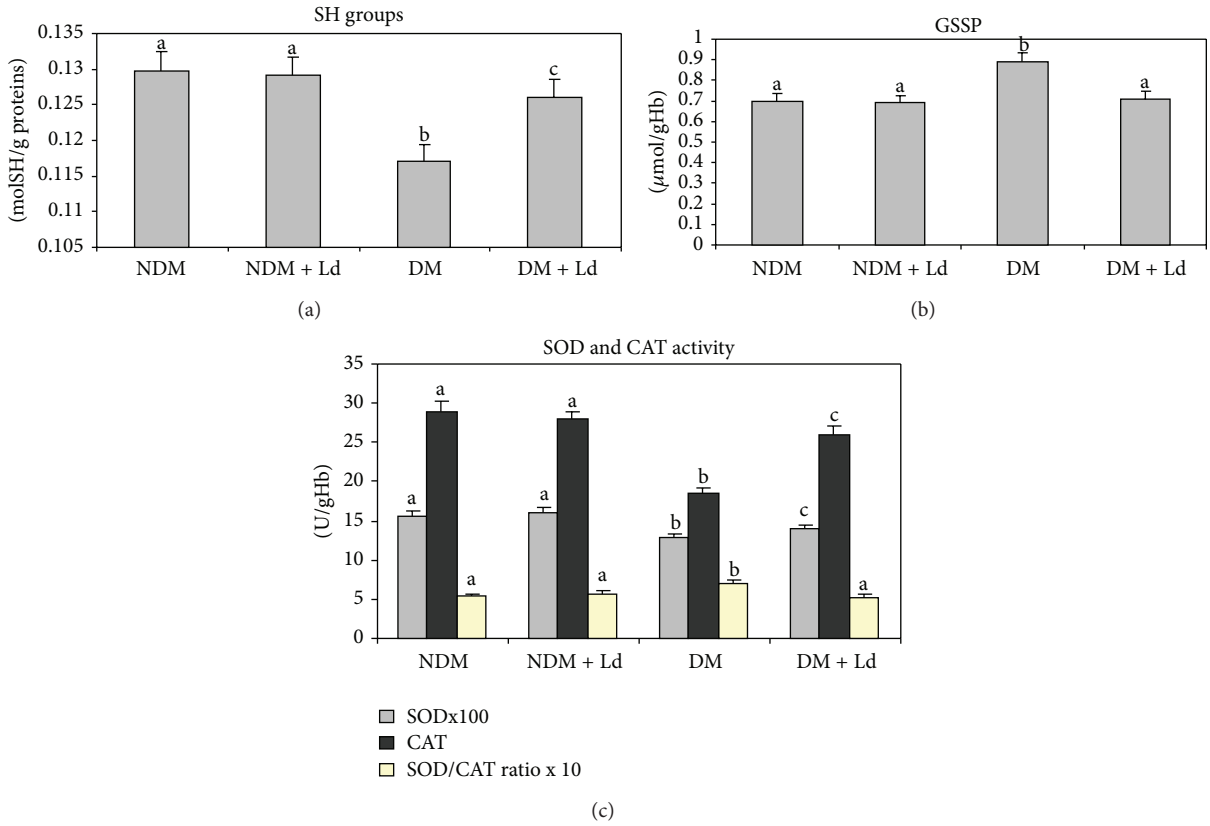


Figure 2: The effect of *L. deterrimus* extract administration on the content of free –SH (a), level of protein bound glutathione (GSSP) (b), and activities of antioxidative enzymes (c) in the circulation. NDM: control rats; NDM + Ld: control rats treated daily with *L. deterrimus* extract for four weeks; DM: STZ-induced diabetic rats; DM + Ld: STZ-induced diabetic rats treated with *L. deterrimus* extract for four weeks. The values are presented as the mean ± S.E.M.; values not sharing a common superscript letter differ significantly at $P < 0.05$.

cells only in diabetic islets and their increased presence in islets from Ld-treated rats. As can be seen in Figure 3, CXCR4-positive immunostaining was only detected in the islets of diabetic rats.

3.4. The Effects of the L. deterrimus Extract Administration on CML-Modified Proteins in Pancreatic Islets. Chronic hyperglycemia causes tissue damage that is mediated in part by the nonenzymatic glycation and oxidation of proteins and lipids and the formation of AGE of which CML is one of the most often used markers [29]. AGE exert their effects through interactions with their receptor RAGE that is normally expressed at low levels on the surface of most cell types. In addition to the circulation, AGE accumulate in tissues where they contribute to the development of diabetic complications. In the pancreas they play a part in progressive β-cell loss [30]. Determination of the fluorescent products of AGE in the serum revealed a 1.5-fold increase in AGE in diabetic rats. In diabetic rats administered Ld, the increase in AGE was at the control level (Figure 4(a)). Immunohistochemical staining revealed an extensive distribution of CML-positive cells in the islets of diabetic rats. In Ld-treated diabetic rats, the CML-positive cells were more disperse (Figure 4(b), CML). RAGE was observed only in the islets of diabetic rats (Figure 4(b), RAGE).

3.5. The Effects of L. deterrimus Extract Administration on the Prosurvival and Proliferative Pathways in Pancreatic Islets. Phosphoinositide 3-kinases (PI3Ks) and its downstream effector protein kinase Akt mediate cellular survival signals that have an essential function in pancreatic β-cell survival [31]. Immunohistochemical staining with activated pAkt shown on Figure 5 revealed its presence in the islets of both control groups, whereas in diabetic islets pAkt-positive staining was weaker. Treatment of diabetic animals with Ld caused a considerable increase in pAkt staining that points to the stimulation of the prosurvival pathway in islets.

Immunohistochemical staining with PCNA (Figure 5), which assumes an essential function in DNA replication and repair, revealed its extensive distribution in the nuclei of islets of both control groups. However, in the islets of diabetic animals, no PCNA immunostaining was detected. The Ld-treated diabetic rats exhibited PCNA staining in their islets (Figure 5, PCNA). The increased presence of PCNA in Ld-treated diabetic rats points to the activation of proliferative mechanisms, which is in agreement with the described stimulation of the prosurvival pathway.

4. Discussion

In this report we have presented evidence for the beneficial effect of the daily i.p. administration for four weeks of the

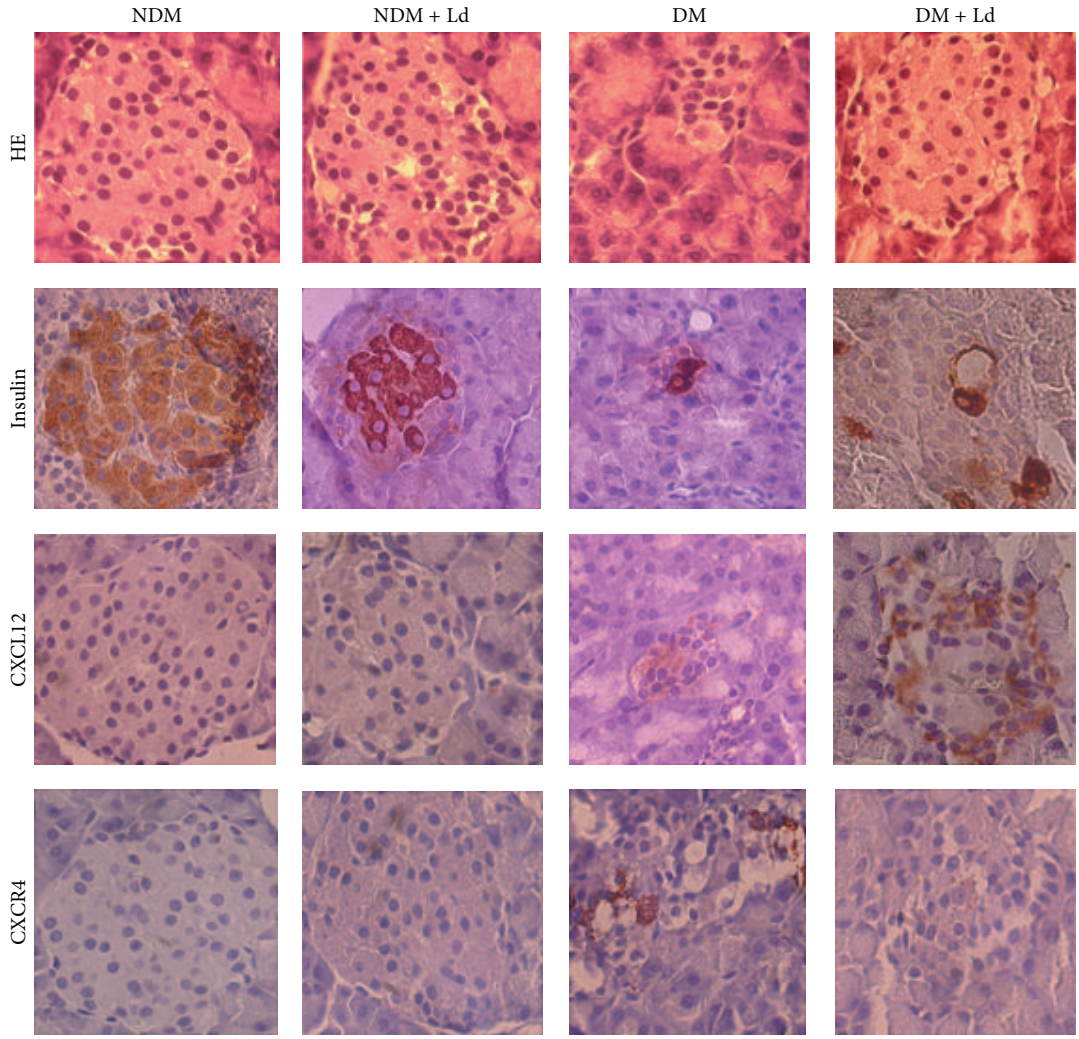


Figure 3: The effect of administration of the *L. deterrimus* extract on histological changes and immunohistochemical localization of insulin and CXCL12 in pancreatic islets. HE: hematoxylin and eosin staining of pancreatic sections; light photomicrographs of insulin, CXCL12, and CXCR4 immunohistochemical staining of pancreatic sections within islets (magnification 40x). NDM: control rats; NDM + Ld: control rats treated daily with *L. deterrimus* extract for four weeks; DM: STZ-induced diabetic rats; DM + Ld: STZ-induced diabetic rats treated with *L. deterrimus* extract for four weeks.

extract of *Lactarius deterrimus* (Ld) to STZ-induced diabetic rats. This was manifested as improved hyperglycemia and a resulting decline in GlyHb, serum protein glycation, and decreased formation of AGE in the circulation, as well as lower triglyceride concentrations. We interpret these global changes as part of a systemic antioxidant effect that impacted pancreatic islets in diabetic rats. By suppressing the formation and accumulation of potential inducers of β-cell damage in diabetic rats (detected as a decrease in CML-containing species in the pancreas), Ld administration activated prosurvival CXCL12/Akt signaling and the proliferative pathway, observed as the increased presence of PCNA-containing β-cells. Therefore, by shifting the balance of β-cell death in favor of β-cell survival, the net result of Ld administration was an increase in the number of functional insulin-positive β-cells.

High concentrations of ROS are involved in pathological changes of cellular functions and disruptions of cellular homoeostasis [1]. In diabetes, persistent hyperglycemia causes enhanced glucose autooxidation and protein glycosylation that increase the production of ROS that are important agents in the development of diabetic complications. The lower activities of the antioxidant enzymes CAT and SOD, along with the decreased protein –SH content and increased amounts of GSSP moieties, serum protein glycation, and GlyHb, revealed the presence of oxidative stress in the circulation of diabetic rats. After the four-week treatment of diabetic rats with Ld, these parameters were practically restored to their physiological values. Ld administration caused a significant decrease in the SOD/CAT ratio that was associated with lower hydrogen peroxide levels, decreased oxidative stress. This shows that the administration of Ld under diabetic conditions attenuated the oxidative stress-induced harmful processes by improving hyperglycemia and promoting increased activities of antioxidative enzymes.

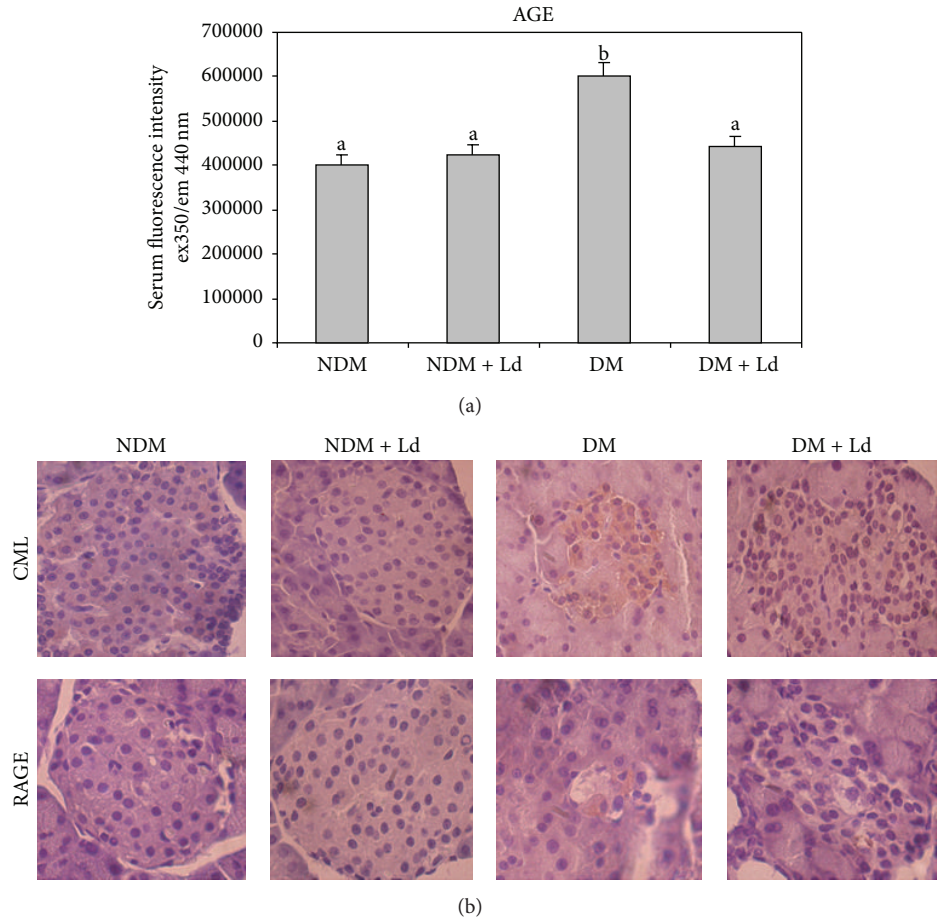


Figure 4: The effect of *L. deterrimus* administration on AGE in the circulation (a) and presence of CML-modified proteins in pancreatic islets (b). Fluorescent products of AGE in the serum (a). Light photomicrographs of immunohistochemical staining for CML and RAGE of pancreatic sections within the islet (b) (magnification 40x). NDM: control rats; NDM + Ld: control rats treated daily with *L. deterrimus* extract for four weeks; DM: STZ-induced diabetic rats, DM + Ld: STZ-induced diabetic rats treated with *L. deterrimus* extract for four weeks. The values are presented as the mean ± S.E.M.; values not sharing a common superscript letter differ significantly at $P < 0.05$.

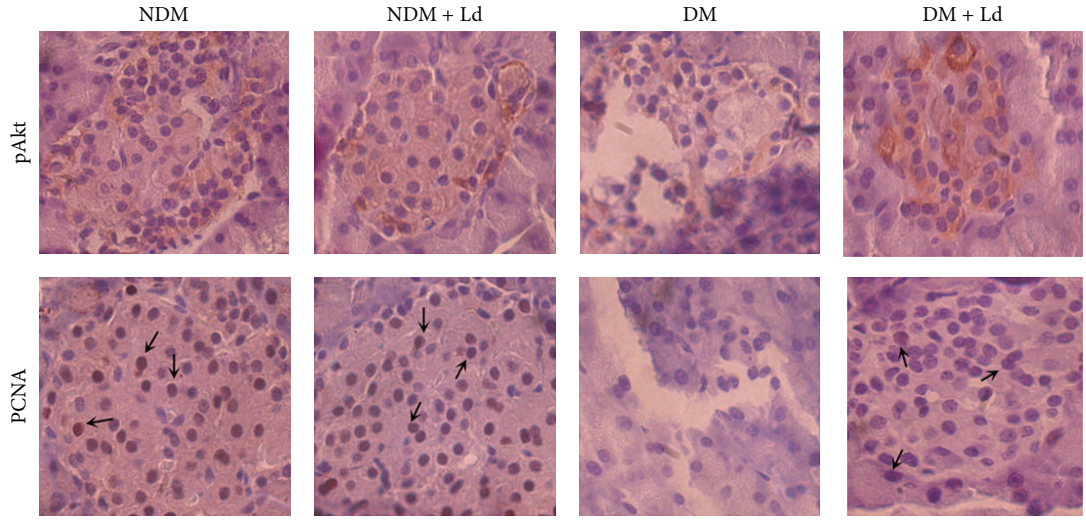


Figure 5: The effect of *L. deterrimus* extract administration on the prosurvival pathway and β-cell proliferation in pancreatic islets. Light photomicrographs of immunohistochemical staining for pAkt and PCNA of pancreatic sections within the islet (magnification 40x). NDM + Ld: control rats treated daily with *L. deterrimus* extract for four weeks; DM: STZ-induced diabetic rats; DM + Ld: STZ-induced diabetic rats treated with *L. deterrimus* extract for four weeks.

These results are consonant with literature data showing that edible mushrooms possess antioxidant and free radical scavenging properties [3]. The Ld applied herein is enriched in both phenolics and flavonoids that contributed to its free-radical scavenging activity (Table 1) [14]. This assumption is in agreement with the generally held view that the antioxidant properties of mushroom extracts correlate with their total content of phenolics [10] and with Wang and Xu [5] who recently provided additional evidence that phenols and polyphenols are the major naturally occurring antioxidant compounds in wild edible mushrooms. We speculate that the free radical scavenging activity of Ld could also be attributed to β-glucan, a principal component of mushrooms known to play important roles in the activation of the nonspecific immune response, reduction of blood cholesterol and blood glucose concentrations, protecting blood macrophages from ionizing radiation, and restoring bone marrow production [5, 32, 33]. Results from our previous work with plant-sourced β glucan revealed its ability to cause a systemic adjustment of redox disturbance and to exert a beneficial effect under diabetic conditions via its antioxidant and anti-inflammatory activities [34, 35].

AGE also play a role in toxic signaling in diabetic pathology by contributing to unbalanced free-radical formation and activating stress signaling in cells through interactions with RAGE. AGE are caused by glycation that involves a series of nonenzymatic reactions between the carbonyl group on reducing sugars and the amino group on proteins [36]. The classical pathway of AGE formation involves a glucose-protein condensation reaction to form Schiff base adducts that undergo Amadori rearrangement [37]. The early glycosylation products accumulate predominantly on long-lived proteins that undergo a series of *in vivo* rearrangements to form irreversible compounds and a number of reactive intermediates that enhance oxidative stress [38]. Our results describe a significant increase in serum AGE in diabetic rats and their reversion to the control level after the Ld treatment. Through the generation of ROS and reactive nitrogen species, AGE contribute to tissue injury by changing the structure and function of proteins, thereby affecting important cellular functions, either directly or via RAGE-activated pathways [39]. In addition to AGE in the circulation, we established the presence of CML, the most abundant AGE *in vivo*, and of RAGE in pancreatic islets of diabetic rats. Importantly, in pancreatic islets of Ld-treated diabetic animals, CML was barely detectable and RAGE was undetectable by the experimental procedure applied herein. AGE generally accumulate in tissues that display diabetic complications [24, 40]. Lee and coworkers [30] have demonstrated that the interaction between AGE and RAGE contributes to the progressive loss of β-cells in diabetes through intracellular ROS generation. This finding and our results indicate that the β-cells of Ld-treated diabetic rats were exposed to a lower level of toxic signals than β-cells of diabetic rats.

The efficacy of medicinal mushrooms in the treatment of diabetes by protecting against β-cell damage through enhanced antioxidant defenses, reduced inflammation, and increased insulin release has been reported [6, 41, 42]. The results obtained herein by immunohistological examination of the pancreas revealed that Ld administration to diabetic rats restrained islet destruction and partially restored the number of insulin-positive cells. The activation and direct involvement of the chemokine CXCL12/CXCR4 receptor pair in cardiomyocytes and pancreatic islets have been demonstrated after tissue injury [43, 44], the latter authors showing increased CXCR4 mRNA and protein expression in insulitis. Recently it was established that injury of β-cells induces CXCL12 expression that initiates the transdifferentiation of α-cells to β-cells in the pancreas [45]. PI 3-kinase and the downstream effector protein Akt play key roles in mediating signals for cell growth, cell survival, cell-cycle progression, and differentiation [31, 46]. Activated, phosphorylated Akt by insulin and growth factors is implicated in glucose metabolism, transcriptional control, and mediation of anti-apoptotic and prosurvival events in β-cells [47, 48]. Yano et al. [49] described the important function of CXCL12 in diabetes attenuation in mice via promotion of β-cell survival by Akt activation. The functions of CXCL12 described in the literature suggested to us that the increased presence of CXCL12 stimulated the Akt pathway, affecting elevated expression of PCNA protein in Ld-treated diabetic rats. This result indicates that Ld exerted a stimulatory effect on islet proliferation and regeneration. Considering the significant capacity of the endocrine pancreas to adjust to changes in insulin demand [50], and that the pancreas contains quiescent cells that can proliferate and replace lost cells [51], we believe that the described stimulatory activity of Ld can exert an important beneficial effect during the initial stage of diabetes development when it is potentially possible to expand the still existing β-cell mass through regeneration. Pancreatic β-cell regeneration induced by β-cell proliferation is often mentioned as a vital goal in the development of effective treatments for diabetes [52]. The findings described herein point to the possibility of developing the therapeutic potential of *Lactarius deterrimus* in diabetes management by employing its ability to alleviate oxidative stress and increase β-cell mass.

5. Conclusion

In vivo antioxidant and antidiabetic effects of the extract from the mushroom *Lactarius deterrimus* were determined. Our results show that administration of the extract to diabetic rats restores antioxidant enzyme activities in the circulation and decreases oxidative stress. Treatment with the extract exhibits a potential for preserving pancreatic islet structure and increasing β-cell mass through activation of the prosurvival CXCL12/Akt pathway and stimulation of β-cell proliferation. We believe that the effects of the mushroom extract are important as it is derived from an established food used for human consumption. However, as a caveat to these findings, the described potentially positive effects have to be proven in human diabetics, and any side effects from prolonged extract administration as described herein need to be assessed.

Conflict of Interests

The authors declared that they have no conflict of interests.

Acknowledgment

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Clinical Study

The Effect of Autologous Platelet-Rich Gel on the Dynamic Changes of the Matrix Metalloproteinase-2 and Tissue Inhibitor of Metalloproteinase-2 Expression in the Diabetic Chronic Refractory Cutaneous Ulcers

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Aim. To investigate the dynamic changes on the expression of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) in the diabetic chronic refractory cutaneous ulcers after the autologous platelet-rich gel (APG) treatment. *Methods*. The study was developed at the Diabetic Foot Care Centre, West China Hospital. The granulation tissues from the target wounds were taken before and within 15 days after APG application. The expression of MMP-2 and TIMP-2 as well as transforming growth factor-β1 (TGF-β1) in the granulation tissue was detected by q TR-PCR and IHC. The relationship between the expression level of MMP-2 and TIMP-2 and their ratio and that of TGF-β1 was analyzed. *Results*. The expression of MMP-2 ($P < 0.05$) was suppressed, and the expression of TIMP-2 ($P < 0.05$) was promoted, while the ratio of MMP-2/TIMP-2 ($P < 0.05$) was decreased after APG treatments. The expression of TGF-β1 had negative correlation with the ratio of MMP-2/TIMP-2 ($P < 0.05$) and positive correlation with the expression of TIMP-2 ($P < 0.05$). *Conclusions*. APG treatment may suppress the expression of MMP-2, promoting that of the TIMP-2 in the diabetic chronic refractory cutaneous wounds. TGF-β1 may be related to these effects.

1. Introduction

Matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases in the degradation of almost all extracellular matrix (ECM), are secreted by keratinocytes and fibroblasts and many other stromal cells. Both collagenases and gelatinases are the most important types, and the latter mainly refer to MMP-2 and MMP-9 and act on collagen type IV-V, gelatin, and so on [1]. The content and activity of MMPs in tissues are regulated by 3 levels of DNA and protein expression, proenzyme activation, and enzyme activity inhibition [2]. Tissue inhibitors of metalloproteinases (TIMPs) take part in the last 2 levels by binding with the fixed sites of the inactive proenzymes or active enzymes and are still influenced by many bioactive substances and cell actions [2, 3]. The known endogenous TIMPs in mammals are classified into 4 types. Of which, TIMP-2 combines with MMP-2 and pro-MMP-2 generally.

It is reported that the dynamic changes on the content and activity of MMP-2 and/or TIMP-2, which are secreted by fibroblasts majorly, play much essential parts in the normal healings, especially during the midterm and later phases, including accelerating revascularization, granulation tissues regeneration as well as the connective tissues reformation, and safeguarding the normal dermis to some extent [4]. The peak of their content overlaps that of granulation tissues regeneration velocity and the high level maintains for 14 days or longer [4].

Owning to the decreased content and/or activity of growth factors and the disturbed balance of MMPs/TIMPs system, which results in excessive solvent activity and then reduced content or damaged structure of the growth factors

and other ECMs locally, the diabetic cutaneous ulcers are always poorly healed. MMP-2 is found to be excessively generated while TIMP-2 is deficiently secreted in diabetic chronic wounds, and the pathologic imbalance may bring about retarded progress of tissue regeneration and revascularization [5].

Within the recent 10 years, the intelligent efficacy of autologous platelet-rich gel (APG) on refractory wounds has been reported [6, 7]. In our preliminary RCT, the cumulative rate of ulcer healing was 95.7% in the APG group versus 56.5% in the standard treatment group ($P = 0.002$); the total effective rate in APG versus standard treatment group was 100.0% versus 73.9% ($P = 0.009$) [8]. The healing-promoting mechanism is recognized including upregulating the content of many growth factors and releasing antibacterial peptides [9–11]. More recently, some platelet-derived wound healing factors (PDWHFs) were reported to influence the MMP-2/TIMP-2 and other pathways in MMPs/TIMPs system in cellular and animal studies [1, 12] and our preliminary clinical experiment further provides some positive evidence about APG treatment on another pathway of MMP-1, MMP-9, and TIMP-1 [13]. However, the data on the effects of APG treatment on the MMP-2/TIMP-2 pathway in diabetic chronic wounds were relatively insufficient.

Furthermore, TGF-β1 has been reported in some basic researches to inhibit the generation of MMP-2 by depressing its genetic transcription and enhance that of TIMP-2 meanwhile [1]. In our preliminary clinical study, the local concentration of TGF-β1 increases 2-3-fold after APG treatment [9]. So TGF-β1 is properly suspected to join in the regulation of APG treatment on MMP-2 and TIMP-2. But so far, no direct evidence has ensured the hypothesis.

Therefore, this study progressively and creatively aimed to investigate the dynamic changes on the expression of MMP-2 and TIMP-2 in the diabetic chronic refractory cutaneous ulcers after the APG treatment as well as their correlation with TGF-β1.

2. Materials and Methods

2.1. Study Design and Population. The study aimed to investigate the dynamic changes on the expression of MMP-2 and TIMP-2 as well as the ratio of MMP-2/TIMP-2 in the diabetic chronic refractory cutaneous ulcers after the APG treatment and then calculate their correlation with TGF-β1. It was carried out at the Diabetic Foot Care Center, West China Hospital, Sichuan University (Sichuan, China) between April 1, 2012, and December 31, 2012. All eligible patients experienced 12-week observation and therapies. The study protocol, informed consent form, and other study related documents were reviewed and approved by the Ethics Committee of West China Hospital.

All eligible patients, screened according to the following inclusion and exclusion criteria, signed the informed consent and prescribed with APG treatment. Those, whose granulation tissues from the target wounds were taken at every observation point before and within 15 days after the first APG application, were taken into analysis. Diabetic patients over 18 years of age, with at least one cutaneous ulcer (area of 1~60 cm^2) or sinus (volume of 1 cm^3 or more) lasting no less than 4 weeks and not meeting an area size reduction of 10–15% per week after at least two-week standard treatments for ulcers, participated in the study [9].

2.2. Treatment Procedures. During the prerecruitment period, all of the participants received systemic therapies and standard care for the cutaneous ulcers. The former consisted of intensive insulin therapy, anti-infection, nerve-trophic and circulation-improving therapies, and so on. The latter composed of topical washing, cleaning, draining, and debridement to remove callous, necrotic tissue and sequestra, as well as dressing changes. Proper limb immobilization and weight offloading were prescribed. Within the treatment period, above-mentioned systemic therapies continued. Participants were prescribed with a topical application of APG upon the wound beds before administration of Suile wound dressing (Hedonist Biochemical Technologies Co., Ltd., USA) and occlusive bandages at baseline. The procedure of APG preparation was described in detail by Yuan et al. [14]: following centrifugation at 313 ×g for 4 minutes, erythrocyte concentrate was removed. PRP and PPP were prepared by centrifugation (1252 ×g) for 6 minutes from the remaining plasma. Thrombin and calcium gluconate were added to PRP, and the gel-like mixture is called APG. Then, Suile dressing and occlusive bandages were maintained for 3 days and then changed at 3-day intervals.

2.3. Evaluations and Endpoints. Wound evaluations were taken along with dressing and bandages changing every 3 days. The granulation tissues within a 5 mm range from the centre of wound bed were also biopsied along with dressing changing and then maintained in the paraformaldehyde or liquid nitrogen for measuring until the area of less than 0.5 cm^2 or volume of less than 0.5 cm^3.

The expression of each target mRNA in the granulation tissue was detected semiquantitatively by quantitative reverse transcription polymerase chain reaction (q TR-PCR). The total RNA was extracted by the Trizol (Roche Dia) method. The high capacity cDNA synthesis kit (TaKaRa Bio Inc.) was applied to carry out the reverse-transcription and generate cDNA. The q RT-PCR (Chromo4, Bio Pad) was carried out by SYBR Green (Roche Dia) method. The primers were synthesized according to related articles by Invitrogen Life Technology Co., Ltd. (Shanghai) (Table 1).

The expression of each target protein in the granulation tissue was locatively and semiquantitatively detected by immunohistochemistry technique (IHC). Immunohistochemistry staining was mediated by ChemMate Envision+HRP/DAB. Primary antibodies were unique: mouse monoclonal antibody of MMP-2 (Abcam), dilution ratio of 1 : 300; rabbit polyclonal antibody of TGF-β1 (Abcam), dilution ratio of 1 : 400; rabbit polyclonal antibody of TIMP-2 (Abcam), dilution ratio of 1 : 100. Secondary antibodies were from common kit for ChemMate Envision+HRP/DAB (Gene Technology Co., Ltd., Shanghai).

Each section was evaluated for the area and density of staining by microscopic examination (×200; BX51; Olympus, Tokyo, Japan). Two noncontiguous microscopic areas were

Table 1: Primer sequences used for the amplification of each target DNA.

Primer	Sequence
TGF-β1 forward	TGGCGATACCTCAGCAACC
TGF-β1 reverse	CTCGTGGATCCACTTCCAG
MMP-2 forward	GGCCCTGTCACTCCTGAGAT
MMP-2 reverse	GGCATCCAGGTTATCGGGGA
TIMP-2 forward	GGCGTTTTGCAATGCAGATGTAG
TIMP-2 reverse	CACAGGAGCCGTCACTTCTCTTG
β-Actin forward	GCGAGAAGATGACCCAGATCATGTT
β-Actin reverse	GCTTCTCCTTAATGTCACGCACGAT

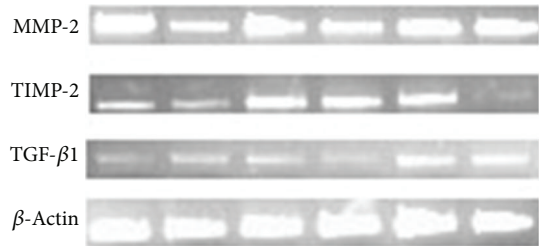

Figure 1: Electrophoretic bands of the q RT-PCR products before and within 15 days after the APG application. Note: the electrophoretic bands of the q RT-PCR products from left to right represent the expression of each target mRNA from the d0, d3, d6, d9, d12, and the d15 one by one.

randomly selected and photographed with a digital camera (DP72; Olympus, Tokyo, Japan). For each selected area, a digital image (1360 × 1024 pixels) was captured and stored as high-resolution image file. To measure the extent of immunoreactivity, computer-aided image analysis software (Image-Pro Plus, Media Cybernetics) was introduced to discriminate the immunostained area, calculate the integrated optical density and immunoreactive area, and count the mean density.

According to our preliminary study, the promoting action of APG on the healing of diabetic chronic ulcer was considered functioning within 15 days after the APG treatment [6]. So, repeated APG treatments were performed every two weeks or 15 days, if necessary. And we analyzed the data within 15 days after the APG treatment.

2.4. Statistical Analysis. Numerical data with normal distribution was represented by mean ± standard deviations (SDs); otherwise, it was represented by median, interquartile range (IQR). Repeated measurement data were tested by general linear model analysis (GLM) and the correlation by Pearson correlation analysis. All tests were two-sided, and P value of 0.05 was considered significant.

3. Results and Discussion

3.1. Results

3.1.1. Epidemiologic Data. A total 51 diabetic inpatients were screened in our centre during this trial and 25 patients were enrolled and prescribed with the APG treatment. Nineteen eligible patients, whose granulation tissues from the target wounds were taken at every observation point before and within 15 days after the first APG application, were taken into analysis, while the rest 6 were excluded for healing too fast to reach the ulcer areas of less than 1 cm^2 before the d15 after the first APG treatment ($n = 3$) or being intolerant to painfulness relevant to the operations ($n = 3$). The 19 patients were aged 43.5 ± 10.1 years, with the diabetic and cutaneous wound duration of 12, 3~16.5 years and 4, 4~14 weeks, respectively. The majority (17, 89.47%) of the participants were male. Each participant maintained normal blood glucose, pressure, and lipid, as well as hepatic and renal functions.

3.1.2. Dynamic Changes on the mRNA Expression of MMP-2, TIMP-2, and TGF-β1 as well as the Ratio of MMP-2/TIMP-2 by qRT-PCR before and after the APG Application. Figure 1 showed the electrophoretic bands of the qRT-PCR products of MMP-2, TIMP-2, TGF-β1, and β-actin and roughly the expression of these genes before and after APG treatment. The mRNA expression of MMP-2 in the granulation tissues decreased within 12 days after the APG application ($P = 0.007$), that of TIMP-2 increased progressively with the peak appearing during the d9–d12 ($P < 0.0001$), and that of TGF-β1 increased with the peak at the d12 ($P = 0.004$) (Figure 2(a)). Meanwhile, the ratio of MMP-2/TIMP-2 decreased with the trough at the d12 after the APG application ($P < 0.0001$) (Figure 2(b)).

3.1.3. The Correlation between the mRNA Expression of TGF-β1 and That of MMP-2, TIMP-2 as well as the Ratio of MMP-2/TIMP-2. The mRNA expression of TGF-β1 had positive correlation with TIMP-2 ($P = 0.028$, $\gamma = 0.680$), no correlation with MMP-2 ($P > 0.05$, $\gamma = -0.071$), and meanwhile negative correlation with the ratio of MMP-2/TIMP-2 ($P = 0.032$, $\gamma = -0.809$) within 15 days after the APG application.

3.1.4. Dynamic Changes on the Protein Expression of MMP-2, TIMP-2, and TGF-β1 by IHC before and after the APG Application. Different shades of brown yellow granules were observed in the immunohistochemical staining sections. It is explained that each target protein expressed differently in ECM and the plasma of positive cells (Figure 3). The dynamic changes of the protein expression by IHC and photodensitometry paralleled with the mRNA expression by q RT-PCR (Table 2, Figure 4), and the differences were all statistically significant after the APG application (MMP-2, $P = 0.001$; TIMP-2, $P < 0.0001$; TGF-β1, $P = 0.0134$).

3.1.5. The Correlation between the Protein Expression of TGF-β1 and That of MMP-2 as well as TIMP-2. The protein expression of TGF-β1 had positive correlation with that of TIMP-2 ($P = 0.041$, $\gamma = 0.660$) and no correlation with MMP-2 ($P > 0.05$, $\gamma = -0.063$) within 15 days after the APG application.

3.2. Discussion. In this study, the mRNA expression of MMP-2, TIMP-2, and TGF-β1 from qRT-PCR technique is similar

Table 2: Dynamic changes of the mean density (MD) of the MMP-2, TIMP-2, and TGF-β1 in granulation tissue before and within 15 days after the APG application (IHC).

	MMP-2	TIMP-2	TGF-β1
Days after APG application			
D0	0.0210 ± 0.0135	0.00282 ± 0.00979	0.00268 ± 0.00192
D3	0.0082 ± 0.0085	0.00404 ± 0.00371	0.00443 ± 0.00850
D6	0.0042 ± 0.0022	0.00931 ± 0.00437	0.00797 ± 0.00118
D9	0.0125 ± 0.0121	0.01368 ± 0.02060	0.00637 ± 0.00731
D12	0.0163 ± 0.0119	0.00991 ± 0.00870	0.00276 ± 0.0672
D15	0.00261 ± 0.00171	0.00210 ± 0.00297	0.00440 ± 0.00291

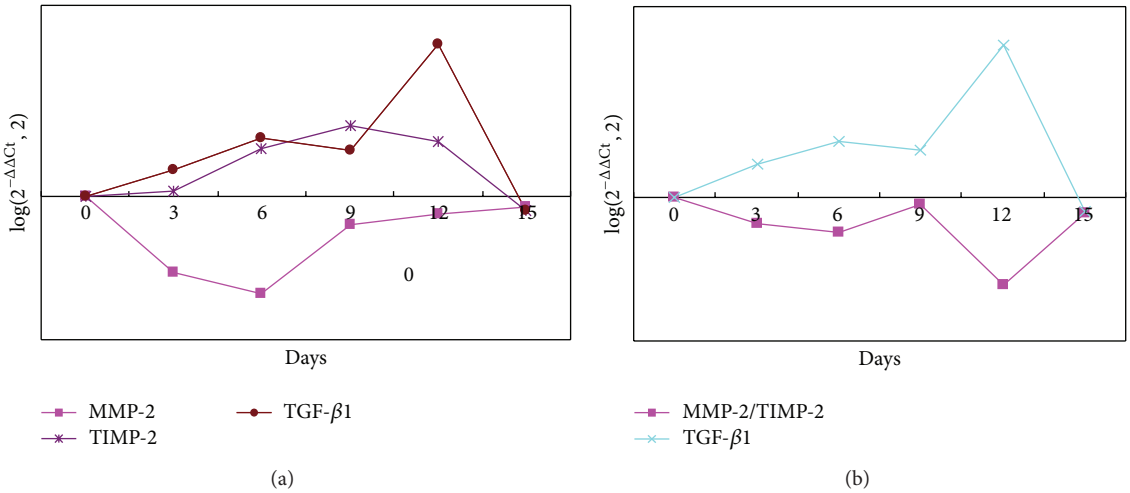

Figure 2: Dynamic changes of the mRNA expression of MMP-2, TIMP-2, and TGF-β1 as well as the ratio of MMP-2/TIMP-2 in the granulation tissue of wounds before and within 15 days after the APG application.

to the protein expression from IHC. The (mRNA and protein) expression of MMP-2 decreases and that of TIMP-2 increases after APG treatment, and the expression of TGF-β1 has positive correlation with that of TIMP-2. Meanwhile, the ratio of MMP-2/TIMP-2, from q RT-PCR, decreases after the APG application and has negative correlation (trend) with the expression of TGF-β1. To sum up, we conclude that APG treatment promotes the expression of TIMP-2, inhibits that of MMP-2, and decreases the ratio of MMP-2/TIMP-2 in diabetic refractory wounds, and TGF-β1 might be related to these effects.

There were some studies referring to the effects of APRP or other PDWHFs on the expression of MMP-2 and/or TIMP-2, but resulting differently. Cáceres et al. [15] reported that APRP stimulated the expression of TGF-β1 and TIMP-2 in gingival fibroblasts; Shin and Oh [16] found that PRP promoted the mRNA expression of MMP-2 and MMP-9 in the wound of OLETF (Otsuka Long-Evans Tokushima fatty rats). Furthermore, Axelrad et al. [17] showed that platelet-activating factors (PAF) excited the mRNA expression of TIMP-2 in the human umbilical vein endothelial cells (HUVEC), but not that of MMP-2. In short, the studies on the MMP-2/TIMP-2 pathway were only a few and almost applying animal and cellular models with simple reaction environment and/or intervenes, while those on the chronic refractory wounds upon APG/APRP treatment, especially clinical trials, were rare. This is before we implemented a clinical trial and investigated the dynamic changes on another pathway (MMP-1 and MMP-9/TIMP-1) of MMPs/TIMPs but not MMP-2/TIMP-2 [13]. So, this study was creatively directed against the MMP-2/TIMP-2 pathway and found a decreased ratio of MMP-2/TIMP-2 upon APG treatment, another potential healing-promoting mechanism of it.

Many basic researches have reported the effects of TGF-β1 on MMPs/TIMPs system and showed its inhibition on MMP-2 expression and promotion to TIMP-2 [1]. This study shows that TGF-β1 might participate in inhibiting MMP-2 expression (negative correlation or trend) while promoting TIMP-2 and further decrease the ratio of MMP-2/TIMP-2, which coincides with the previous basic finds. It ensures the previous hypothesis of TGF-β1 mediating the action of APG and provided clinical data on the effects of TGF-β1 on the pathway of MMP-2.

Previously, no studies (especially the clinical) aimed to show the effects of the APG/PRP treatment on the dynamic changes of MMP-2/TIMP-2 pathway. We make up the deficiency in the dependent field and initiate further exploring. However, we do not set up a controlled group to be compared with and do not analyze a relatively small sample size; so further work is necessary.

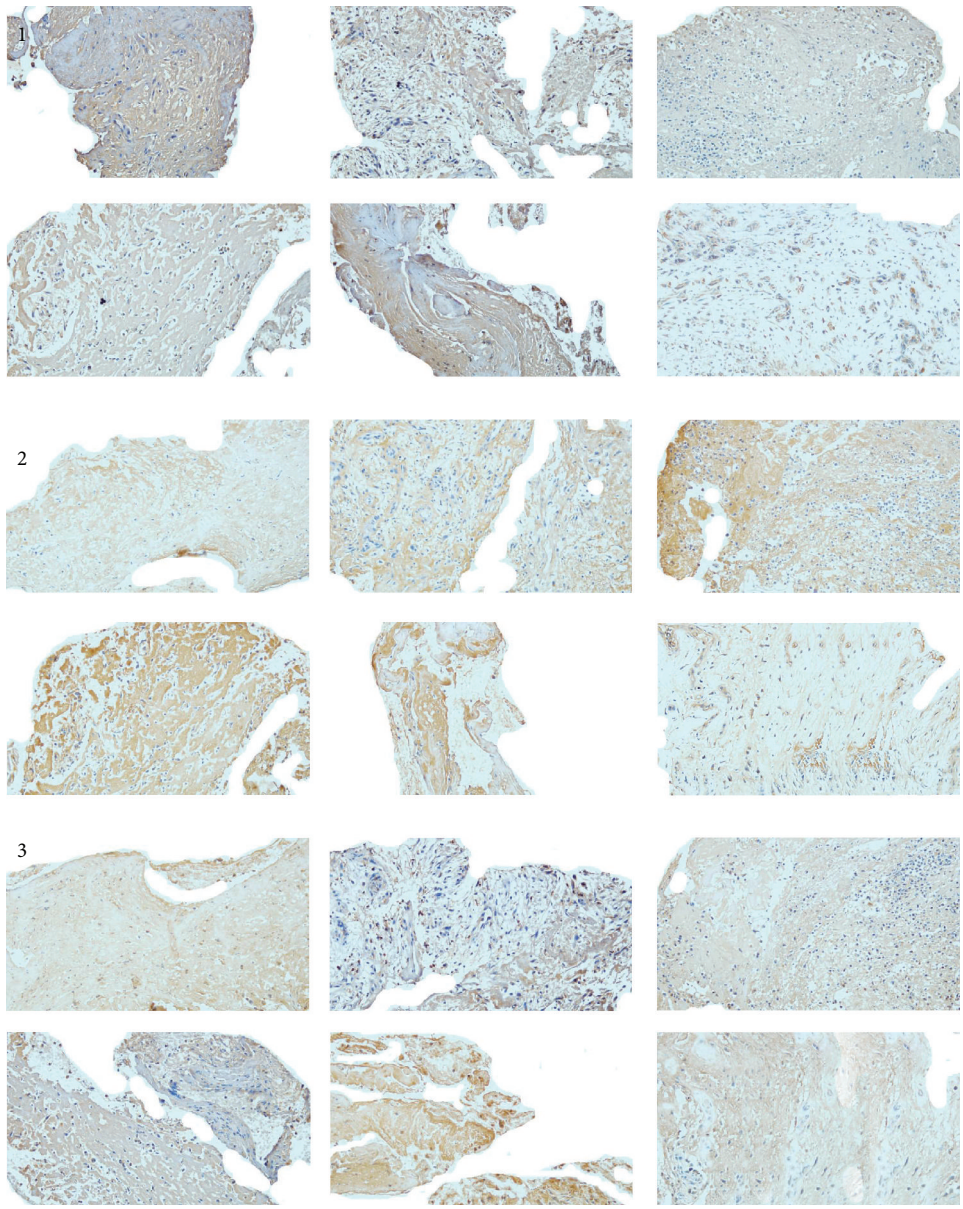

Figure 3: Dynamic changes on the expression of each target protein in granulation tissue before and within 15 days after the APG application. Note: the images captured from sections present the dynamic changes of each target protein within 15 days after the first APG application. The immunoreactants in the images from number 1 to 3 were MMP-2, TIMP-2, and TGF-β1 one by one. Each number of images includes 6 parts, which were taken at the d0, d3, d6, d9, d12, and d15 from left to right. The expression of MMP-2 was suppressed after APG treatment and that of TIMP-2 was promoted by APG treatment, and levels at each observation point were higher than baseline before the d12, with the peak appearing at about the d9–12. TGF-β1 was promoted also, and the peak came out at the d12.

Turning the healing microenvironment of chronic wounds into the acute or acute-like ones, through positive local treatment, is a key point to overcome the challenge of chronic refractory wound. Balancing the ratio of MMPs/TIMPs is an important way to improve the healing condition and accelerate the healing velocity for diabetic wounds. MMP-2/TIMP-2 pathway is a crucial ingredient of MMPs system acting in, especially the midterm and later phases of healing, and necessary for the granulation tissues regeneration and the connective tissues reformation and so on. Actually, it is worthwhile to focus on not only the APG treatment but also all the new local therapies.

4. Conclusions

APG treatment may suppress the expression of MMP-2 and promote that of TIMP-2 in the diabetic chronic refractory cutaneous wounds and furthermore decrease the ratio of the MMP-2/TIMP-2. TGF-β1 may be related to these effects.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

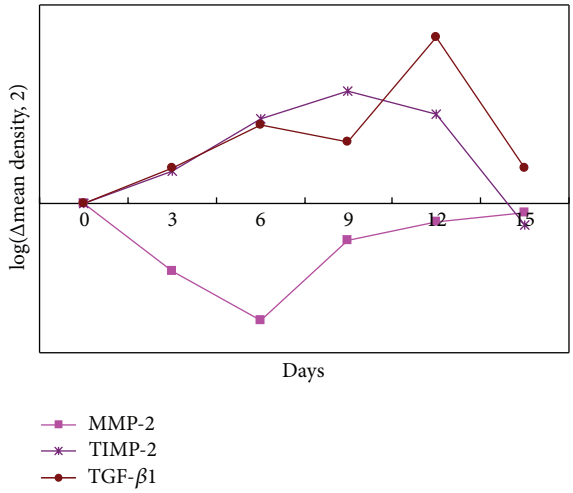

Figure 4: Dynamic changes of the protein expression of MMP-2, TIMP-2, and TGF-β1 before and within 15 days after the APG application. Dynamic changes of the MMP-2, TIMP-2, and TGF-β1 before and within 15 days after the first APG application.

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Research Article

The Influence of Peripheral Neuropathy, Gender, and Obesity on the Postural Stability of Patients with Type 2 Diabetes Mellitus

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Aim. To assess the influence of peripheral neuropathy, gender, and obesity on the postural stability of patients with type 2 diabetes mellitus. *Methods.* 151 patients with no history of otology, neurology, or orthopaedic or balance disorders accepted to participate in the study. After a clinical interview and neuropathy assessment, postural stability was evaluated by static posturography (eyes open/closed on hard/soft surface) and the "Up & Go" test. *Results.* During static posturography, on hard surface, the length of sway was related to peripheral neuropathy, gender, age, and obesity; on soft surface, the length of sway was related to peripheral neuropathy, gender, and age, the influence of neuropathy was larger in males than in females, and closing the eyes increased further the difference between genders. The mean time to perform the "Up & Go" test was 11.6 ± 2.2 sec, with influence of peripheral neuropathy, gender, and age. *Conclusion.* In order to preserve the control of static upright posture during conditions with deficient sensory input, male patients with type 2 diabetes mellitus with no history of balance disorders may be more vulnerable than females, and obesity may decrease the static postural control in both males and females.

1. Introduction

Intact balance is required to maintain postural stability as well as to assure safe mobility during activities of daily life. Balance corrections imply the interaction among several sensory inputs and the major contributor during quiet upright stance may be somatosensory inputs [1, 2]; information from the legs is utilized for both direct sensory feedback and use of prior experience in scaling the magnitude of automatic postural responses [3].

A frequent cause of peripheral neuropathy is type 2 diabetes mellitus [4]. In this group of patients, the frequency of balance symptoms may be related to both the time elapsed since the diabetes was diagnosed and the history of peripheral neuropathy and retinopathy [5]. Assessment of postural control during upright stance has shown that patients with diabetes and peripheral neuropathy may sway more than those without peripheral neuropathy [6–8]. In addition, men may exhibit more spontaneous sway than women [9] and adults with obesity may have a decrease in postural stability with a larger dependency on vision to control balance [10]. Increased body mass may produce instability [11]; subjects with a body mass index greater than 30 maintain shorter times in balance and longer times unbalanced as compared

Table 1: Mean and standard deviation of the mean of the age of 151 patients with type 2 diabetes mellitus, with the number of males/females and the frequency of peripheral neuropathy, according to the BMI group.

Variables	BMI < 30 (n = 92)	BMI 30 to <3 5 (n = 42)	BMI ≥35 (n = 17)
Age (mean ± S.D.)	59 ± 9.2	55.4 ± 9.4	51.6 ± 8.1
Males/females	26/66	12/30	6/11
Peripheral neuropathy (%)	14.1%	33.3%	29.4%

with lean individuals [12]. Even further, in subjects with obesity, weight loss seems to improve measures of static postural stability [13].

The aim of this study was to assess the influence of peripheral neuropathy, gender, and obesity on the postural stability of patients with type 2 diabetes mellitus, receiving primary health care.

2. Patients and Methods

After the study was approved by the institutional research and ethics committee, 151 consecutive patients with type 2 diabetes mellitus receiving primary health care gave their informed consent to participate. None of them were seeking medical care due to balance decline or had history of otology, neurology, psychiatry, or orthopaedic or balance disorders. All of them denied receiving ototoxic medication. Patients were aged 38 to 80 years (mean 57.1 ± S.D. 9.4 years), 107 were females (57 ± 8.8 years old), and 44 were males (57.5 ± 11.1 years old); 48% (95% C.I. 38.1–57.9%) of them had systemic high blood pressure. The mean age when diabetes was diagnosed was 47.7 ± 10 years and the time elapsed since diabetes was diagnosed was 9.3 ± 5.7 years. Their mean glucose serum level was 146.7 ± 55.2 mg/100 mL, and 64.8% (95% C.I. 57.2–72.4%) of them had HbA1c >7%; the most frequent medication was metformin (86%, 95% C.I. 80.5–91.5%) and 22% (95% C.I. 15.4–28.6%) of the patients required insulin.

The mean body mass index (BMI) of the patients was 29 ± 4.8. However, 92 patients had a BMI <30 (59 ± 9.2 years old), 42 patients had a BMI from 30 to <35 (55.4 ± 9.4 years old), and 17 had a BMI ≥35 (51.6 ± 8.1 years old).

Peripheral neuropathy was evaluated at first by the Michigan Diabetic Neuropathy Score [14] and the Semmes-Weinstein 10 g monofilament; when any of these two instruments were positive, nerve conduction studies were performed (Spirit, Nicolet, Madison) [15].

Postural stability was evaluated by body sway during static posturography and the timed "Up & Go" test [16]. Body sway during quiet upright stance was recorded at 40 Hz using a force platform (Posturolab 40/16 Medicapteurs, Cedex); each trial lasted for 51.2 sec and, during this period, subjects were asked to stand upright and barefoot on the platform as still as possible with arms at their sides. Recordings were made under 4 conditions, while adding or not a layer of foam rubber (5 cm thick, density of 2.5 pcf) to the base of support, with the eyes either open or closed [17]: condition 1 = hard surface and eyes open; condition 2 = hard surface and eyes closed; condition 3 = soft surface and eyes open; condition 4 = soft surface and eyes closed. Before each trial, the feet were positioned according to the manufacturer reference, and small adjustments were made online; recordings with the eyes closed were obtained just after acquiring the data with the eyes open, without moving the feet. To perform the "Up & Go" test, patients were asked to stand from a chair with armrests, walk 3 meters, turn, and go back to their seat at their normal pace. A standard digital stopwatch was used to record the time to the nearest tenth of a second, from the command to "go" to the time when the backsides of the patient touched the chair.

Statistical analysis was performed using t-test and analysis of covariance. The significance level was set at 0.05. To perform the analysis of covariance, the BMI of the patients was classified as follows: <30, 30 to <35, and ≥35; the age and the number of males/females according to the BMI group are described in Table 1.

3. Results

Peripheral neuropathy was diagnosed in 32 patients (21.2%, 95% C.I. 14.7–27.7) who had a similar age compared to patients without peripheral neuropathy (56.7 ± 10.5 years old versus 57.2 ± 9.2) but had a longer time of evolution of the disease (11.8 ± 6.2 years versus 8.6 ± 5.5 years) (t-test; $P = 0.005$). A motor component was evident only in 3 patients. The percentage of patients with peripheral neuropathy according to the BMI group is described in Table 1, which was more frequent in those with a BMI ≥30, but with no significant difference among the subgroups.

The characteristics of sway are described in Table 2. Since the area of sway showed high variability among groups and conditions, significant results were observed only in the length of sway (MANCoVA, $P < 0.05$), with an influence of the following variables:

(i) condition 1 (multiple $R = 0.5$; $P < 0.001$): the gender (beta = 0.41, 95% C.I. 0.05–0.77) (Figure 1) and the age of the patients (beta = 0.36, 95% C.I. 0.21–0.52), with no significant interactions;

(ii) condition 2 (multiple $R = 0.44$; $P = 0.001$): the gender (beta = 0.66, 95% C.I. 0.29–1.03) (Figure 1), the BMI group (beta = 0.58, 95% C.I. 0.14–1.01), and the evidence of peripheral neuropathy (beta = 0.42, 95% C.I. 0.08–0.77); an interaction between the BMI group and the evidence of peripheral neuropathy was observed (beta = 0.58, 95% C.I. 0.11–1.06);

Table 2: Mean and standard deviation of the mean of the sway characteristics of 151 patients with type 2 diabetes mellitus during upright stance on hard/soft surface, with the eyes open/closed.

Variables	BMI < 30 (n = 92)		BMI 30 to < 35 (n = 42)		BMI ≥ 35 (n = 17)	
	Eyes open	Eyes closed	Eyes open	Eyes closed	Eyes open	Eyes closed
	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
Hard surface						
Length (mm)	330 ± 104	448 ± 219	347 ± 126	481 ± 292	332 ± 99	522 ± 243
Area (mm^2)	105 ± 99	215 ± 671	108 ± 82	198 ± 295	139 ± 112	280 ± 267
X[a] position (mm)	4.5 ± 36.8	0.75 ± 6.3	0.09 ± 6.2	0.69 ± 6.2	0.32 ± 4.8	0.67 ± 4.7
Y[b] position (mm)	−29.6 ± 19.1	−28.2 ± 15.5	−34.6 ± 19.5	−32.4 ± 18.5	−36.8 ± 14.2	−33.1 ± 12.1
Soft surface						
Length (mm)	447 ± 189	686 ± 308	452 ± 197	724 ± 385	368 ± 91	652 ± 252
Area (mm^2)	204 ± 283	413 ± 505	216 ± 210	505 ± 521	234 ± 229	420 ± 361
X[a] position (mm)	−0.01 ± 7.2	−0.68 ± 7.6	2.12 ± 6.4	1.06 ± 6.4	−1.59 ± 4.3	−1.82 ± 5.7
Y[b] position (mm)	−26.6 ± 17.5	−25.3 ± 15.9	−33.2 ± 20.9	−29.5 ± 24.5	−32.9 ± 14	−31.2 ± 13.9

[a] X: lateral-lateral position of the centre of pressure.
[b] Y: anterior-posterior position of the centre of pressure.

(iii) condition 3 (multiple R = 0.52, P < 0.01): the gender (beta = 0.54, 95% C.I. 0.19–0.89) (Figure 1), the evidence of peripheral neuropathy (beta = 0.35, 95% C.I. 0.02–0.68), and the age (beta = 0.35, 95% C.I. 0.19–0.50), with no significant interactions;

(iv) condition 4 (multiple R = 0.47; P < 0.001): the gender (beta = 0.74, 95% C.I. 0.38–1.11) (Figure 1), the evidence of peripheral neuropathy (beta = 0.46, 95% C.I. 0.12–0.80), and the age (beta = 0.2, 95% C.I. 0.05–0.36); an interaction between the gender and the evidence of peripheral neuropathy was observed (beta = 0.40, 95% C.I. 0.01–0.79).

These results were consistent when comparing the recordings with the eyes open or closed, either on hard or on soft surfaces as follows.

(i) *Hard Surface*. There was an influence of (multiple R = 0.53; P < 0.001) the gender (beta = 0.51, 95% C.I. 0.27–0.76), the BMI group (beta = 0.38, 95% C.I. 0.09–0.67), the evidence of peripheral neuropathy (beta = 0.3, 95% C.I. 0.07–0.59), and the age (beta = 0.17, 95% C.I. 0.06–0.28); an interaction between the BMI group and the evidence of peripheral neuropathy was observed (beta = 0.34, 95% C.I. 0.2–0.65) (Figure 2).

(ii) *Soft Surface*. There was an influence of (multiple R = 0.62; P < 0.001) the gender (beta = 0.58, 95% C.I. 0.35–0.8), the evidence of peripheral neuropathy (beta = 0.36, 95% C.I. 0.15–0.57), and the age (beta = 0.12, 95% C.I. 0.12–0.32); an interaction between the gender and the evidence of peripheral neuropathy was observed (beta = 0.32, 95% C.I. 0.08–0.56).

The mean time to perform the "Up & Go" test was 11.6 ± 2.2 sec. Analysis of covariance showed that the time to perform the test had an influence of (multiple R = 0.3; P < 0.001) peripheral neuropathy (beta = 0.23, 95% C.I. 0.018–0.045), the gender (beta = 0.16, 95% C.I. 0.018–0.32), and the age (beta = 0.17, 95% C.I. 0.01–0.33).

4. Discussion

The results of this study show that, in patients with type 2 diabetes mellitus, during upright stance, the influence of peripheral neuropathy and vision on the length of sway may be more evident in male than in female patients, particularly while standing on a soft surface, and obesity may have a further influence on sway in the two genders, while standing on a hard surface, when vision is not available. However, when performing a standardized daily-life task "Up & Go test", the influence from obesity may not be evident, even when peripheral neuropathy and the gender have an influence.

To maintain stability when moving from one sensory context to another, it is important to reweigh the sensory information depending on the context. In healthy subjects, increased severity of experimentally induced loss of plantar cutaneous sensitivity may be associated with greater postural sway; such an association could be affected by the availability of visual input and the size of the support surface [18]. In this study, we observed that, in patients with type 2 diabetes mellitus, the influence of peripheral neuropathy on the length of sway is related to the gender and to obesity, with larger sway in male than in female patients and a larger increase of sway after closing the eyes in obese subjects than in nonobese subjects.

In the present study, in patients with peripheral neuropathy, static posturography showed that closing the eyes while standing on hard surface had a larger effect in patients with a BMI ≥30 than in those with a BMI <30, and while standing on a soft surface, patients with a BMI ≥35 had less sway than patients with a BMI <35. In contrast, an independent

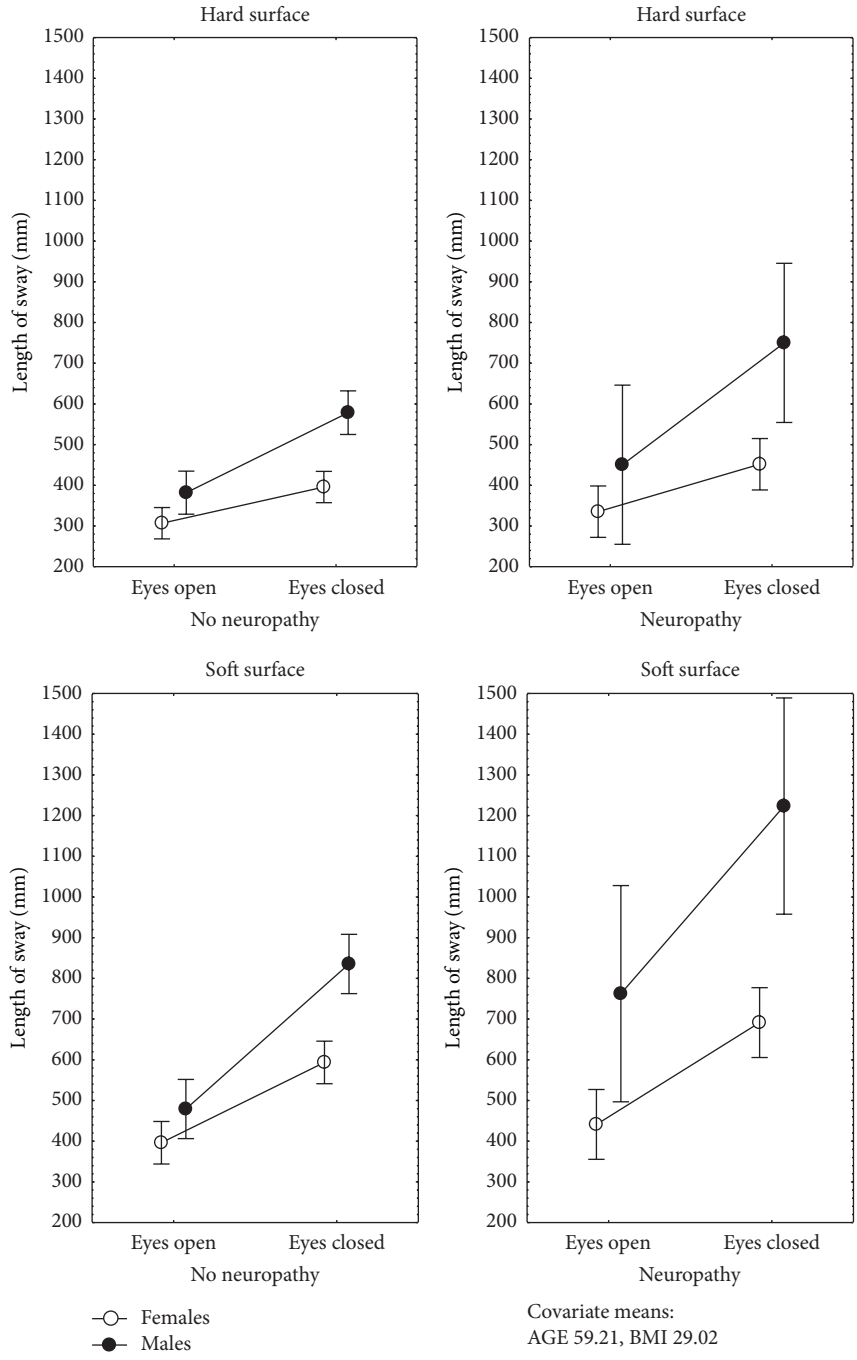

Figure 1: Mean and standard error of the mean of the length of sway during static posturography, by gender, evidence of neuropathy, and sensory condition of 151 patients with type 2 diabetes mellitus receiving primary health care.

influence of neuropathy was observed during all the sensory conditions of the study. Although peripheral neuropathy was more frequent among patients with a BMI ≥30, it was similar among those with a BMI from 30 to <35 and those with a BMI ≥35 (Table 1). In subjects without diabetes mellitus, evidence has shown that, during quiet standing, obese subjects have an increase of the peak pressure on fore-foot and plantar ground contact area [19], and compared to control and overweight subjects obesity may be related to a decrease in postural stability, when vision is not available, suggesting that obese subjects may be more dependent on vision to control balance [10]. Additionally, evidence has shown that, after closing the eyes, the increase of sway in obese subjects may be similar when recordings are made either on hard or on soft surface [10], suggesting that obese subjects may use their somatosensation to control posture differently than lean and

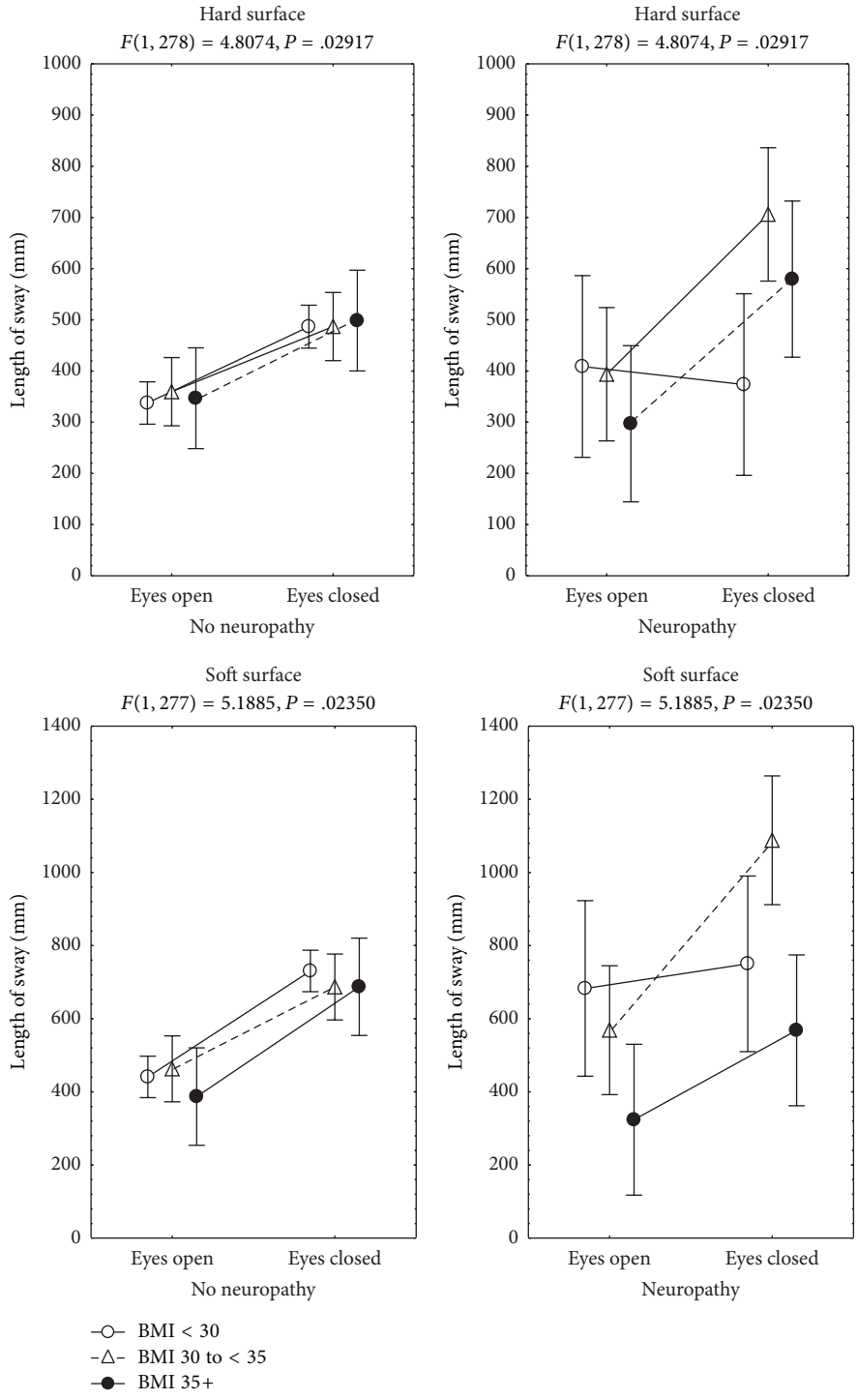

Figure 2: Mean and standard error of the mean of the length of sway during static posturography, by body mass index group, evidence of neuropathy, and sensory condition of 151 patients with type 2 diabetes mellitus receiving primary health care.

overweight subjects, which is consistent with the results of the present study.

Although the influence of age was evident during all the sensory conditions and the "Up & Go" test, it is already known as well that sway increases with increasing age [20, 21], with an increased dependence on vision [22, 23]; the results of this study suggest that the influence of peripheral neuropathy, obesity, and gender on the length of sway may be not dependent on age. Since male patients had a similar age compared to female patients, patients with peripheral neuropathy, as well,

had a similar age compared to patients without neuropathy and patients with obesity were even younger than nonobese patients (Table 1).

The finding of a larger sway in males than in females is consistent with previous reports showing that men may exhibit more spontaneous sway than women, and this difference may increase when there is no visual input [9, 10]. In addition, in this study we observed that the difference between genders may be increased by peripheral neuropathy and by distortion of somatosensory inputs (soft surface conditions).

The findings of this study should be interpreted in the context of its limitations. Since the study has a cross-sectional design, imprecision of any real association may be possible. The enrolment was limited to patients requiring primary health care, so the results may not apply to patients with more physical impairments.

5. Conclusion

In order to preserve the control of static upright posture during conditions with deficient sensory input, male patients with type 2 diabetes mellitus with no history of balance disorders may be more vulnerable than females and, in both males and females, obesity may decrease their static postural control.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Role of MicroRNAs in Diabetic Nephropathy

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Diabetic nephropathy (DN), as one of the chronic complications of diabetes, is the major cause of end-stage renal disease. However, the pathogenesis of this disease is not fully understood. In recent years, research on microRNAs (miRNAs) has become a hotspot because of their critical role in regulating posttranscriptional levels of protein-coding genes that may serve as key pathogenic factors in diseases. Several miRNAs were found to participate in the pathogenesis of DN, while others showed renal protective effects. Therefore, targeting miRNAs that are involved in DN may have a good prospect in the treatment of the disease. The aim of this review is to summarize DN-related miRNAs and provide potential targets for diagnostic strategies and therapeutic intervention.

1. Introduction

As one of the most important long-term complications of diabetes, diabetic nephropathy (DN) is the major cause of end-stage renal disease [1] and high mortality in diabetic patients. The main clinical features of DN are persistent albuminuria and progressively declined glomerular filtration rate (GFR). Microalbuminuria (30–300 mg a day of albumin in urine) indicates early DN while macroalbuminuria (>300 mg/day) represents DN progression [2]. The major pathological features of DN are characterized by hypertrophy and expansion in the glomerular mesangium and tubular compartments, along with podocyte dysfunction and accumulation of extracellular matrix (ECM) proteins. Several mechanisms, including hyperglycemia, advanced glycation end products, protein kinase C, oxidative stress, inflammation, and poly(ADP-ribose) polymerase activation, are believed to contribute to the pathogenesis and development of DN [3]. Several typical cell signaling pathways have been proven to be involved in DN. For example, transforming growth factor-β (TGF-β) is a well-known pathway leading to the accumulation of ECM in DN [4–6]. Phosphoinositide 3-kinase-protein kinase B (PI3K-Akt) pathway is considered to result in glomerular hypertrophy and ECM accumulation [7, 8]. Mitogen-activated protein kinase (MAPK) family including P38, extracellular signal-regulated kinases (ERK), and c-Jun N-terminal kinases pathways are also found to cause DN [9–13]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a key inflammatory pathway, recruits a variety of inflammatory cytokines involved in DN [14–16]. However, the molecular pathogenesis hidden behind is still not fully understood.

MicroRNAs (miRNAs) are endogenously produced short noncoding RNAs of about 21–25 nucleotides that have been shown to play important roles in modulating gene expression, thus affecting almost every key cellular function [17, 18]. The biogenesis of miRNA has been largely understood and the canonical pathway was summarized in Figure 1.

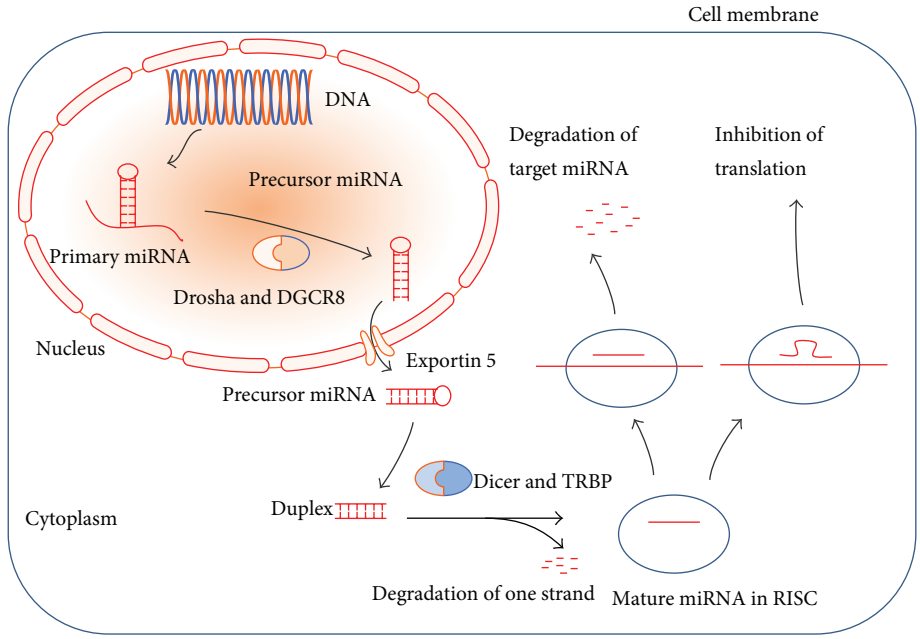

Figure 1: Biogenesis of miRNA. miRNAs are transcribed from DNA into primary-miRNAs (Pri-miRNAs) which contain hairpin-like structures. RNase III Drosha and its binding partner, DiGeorge syndrome critical region gene 8 (DGCR8), bind to the hairpin structures in Pri-miRNAs and process them into precursor miRNAs (Pre-miRNAs). Through Exportin 5, Pre-miRNAs are transferred into cytoplasm and are processed by another RNase III enzyme, Dicer, in collaboration with transactivating response RNA-binding protein (TRBP) to generate the mature miRNA duplex. One strand of the duplex goes into RNA-induced silencing complex (RISC), while the other is degraded. In RISC, mature miRNA recognizes target mRNAs through sequence complementarity, resulting in either degradation of the target mRNA (perfect complementarity to 3′UTR) or more frequently inhibition of translation (imperfect complementarity to 3′UTR).

The final destiny of miRNAs is degradation in processing bodies [19–21]. It is estimated that about 60% of the human protein-coding genes can be targeted by miRNAs. Thus, research on miRNAs has attracted a high level of interest. Accumulating evidence has demonstrated that miRNAs are found to regulate signaling pathways involved in the pathogenesis of DN. For example, miR-192 targeted zinc finger E-box binding homeobox 1/2 (ZEB1/2) to activate TGF-β signaling pathway, leading to renal fibrosis proteinuria [22]. miR-21 targeted phosphatase and tensin homolog (PTEN) to induce the overactivation of Akt signaling pathway, followed by renal fibrosis and hypertrophy [23]. These DN-inducing miRNAs were found to be overexpressed in diabetic kidney, contributing to the pathogenesis of DN. In contrast, downregulated miRNAs showed renal-protective effects. Thus, we briefly summarize previous work by classifying the DN-related miRNAs into two groups, the upregulated (Table 1) and the downregulated (Table 2) classification of miRNAs, with the aim of providing a clear profile of DN-related miRNAs suggesting potential targets not only for diagnosis but also for therapeutic intervention.

2. Upregulated miRNAs in DN

Under diabetic conditions, several miRNAs are upregulated in diabetic kidney. These miRNAs bind to the 3′UTR of renoprotective genes which leads to their decreased expression. As a result, these upregulated miRNAs contribute to the pathogenesis of DN (Table 1).

2.1. miR-192. The pioneering work on miR-192 by Kato and coworkers indicated a central role of miR-192 in the development and progression of DN [22, 24, 25]. miR-192 was upregulated along with increased mRNA level of collagen 1 alpha 2 (COL1α2) compared with nondiabetic control in glomeruli isolated from streptozotocin- (STZ-) induced type 1 diabetic mice and db/db type 2 diabetic mice. Importantly, miR-192 was found to repress δEF1 and Smad-interacting protein 1, which are repressors of COL1α1 and COL1α2 [24]. Another study showed miR-192-miR-200 cascade induced TGF-β1 expression. Thus, miRNA-regulated circuits may amplify TGF-β1 signaling, accelerating DN [22]. More recently, the same group found that TGF-β induced acetylation of chromatin and Ets-1 to alleviate repression of miR-192 in DN. The induction of miR-192 expression by TGF-β in mouse mesangial cells (MMCs) initially involved the Smad transcription factors, followed by sustained expression that was promoted by acetylation of the transcription factor Ets-1 and of histone H3 by the acetyltransferase p300 [25].

Putta et al. treated STZ-induced diabetic C57 mice with locked nucleic acid (LNA) modified anti-miR-192 and observed significantly increased ECM repressor ZEB1/2

Table 1: Upregulated miRNAs.

miRNAs	Targets	Biological outcome	Models	References
miR-192	δEF1, SIP1	COL1α1 and COL1α2 ↑	Diabetic mice (STZ), db/db mice	[24]
	ZEB1/2	TGF-β, Col, FN ↑, proteinuria ↓	Diabetic mice (STZ)	[22]
miR-216a	PTEN, YBX1	MMC survival, hypertrophy, COL1α2 ↑	MMCs	[28, 29]
miR-217	PTEN	MMC survival, hypertrophy	MMCs	[28]
miR-200b/c	ZEB1	TGF-β1, COL1α2, COL4α1 ↑	Diabetic mice (STZ), db/db mice, MMCs	[22]
	FOG	p-Akt, ERK ↑, hypertrophy	Diabetic mice (STZ), MMCs	[30]
miR-21	PTEN, PRAS40	p-Akt, mTORC1, hypertrophy, COL1α2, FN ↑	HMCs	[23]
	SMAD7	Microalbuminuria, TGF-β, NF-κB ↑	db/db mice	[34]
	TIMP3	TIMP3 ↓	Diabetic mice (STZ), MMCs, kidney biopsy (human)	[35]
	TIMP1	COL4, FN, ACR ↑; CCR ↓	kk-ay mice	[37]
miR-377	PAK1, SOD	FN ↑	Diabetic mice (STZ), MMCs, HMCs	[38]
miR-195	BCL2	Caspase-3, caspase-8 ↑	Diabetic mice (STZ), podocytes, MMCs	[42, 43]
miR-215	CTNNBIP1	β-Catenin, FN, α-SMA ↑		[49]
miR-124	INTEGRIN$\alpha3\beta1$	Urinary podocyte nephrin, podocin, albumin ↑	Diabetic rats (STZ)	[51]
miR-29c	SPRY1	Albuminuria, ECM ↑	db/db mice	[59]
miR-1207-5p		TGF-β1, PAI-1, FN ↑	HK-2 cells, podocytes, normal mesangial cells	[52]
miR-135a	TRPC1	Microalbuminuria ↑, renal fibrosis ↑	db/db mice	[53]

STZ: streptozotocin; δEF1: deltaEF1 (ZEB1); FOG: Friend of GATA; SIP1: Smad-interacting protein 1; Col: collagen; ZEB1/2: zinc finger E-box binding homeobox 1/2; YBX1: Y box binding protein 1; α-SMA: alpha smooth muscle actin; PTEN: phosphatase and tensin homolog; p-Akt: phosphorylated protein kinase B; PRAS40: proline-rich Akt substrate 40; mTORC1: mechanistic target of rapamycin complex 1; SMAD3: mothers against decapentaplegic homolog 3; SMAD7: mothers against decapentaplegic homolog 7; TIMP: tissue inhibitors of metalloproteinase; PAK1: p21 activated kinase; SOD: superoxide dismutase; BCL2: B-cell CLL/lymphoma 2; INTEGRIN$\alpha3\beta1$: integrin alpha 3 beta 1; SPRY1: Sprouty homolog 1; NF-κB: nuclear factor kappa B; TGF-β: transforming growth factor beta; ERK: extracellular signal-regulated kinases; ECM: extracellular matrix; FN: fibronectin; PAI-1: plasminogen activator inhibitor-1; MMC: mouse mesangial cell; HMC: human mesangial cell; RMC: rat mesangial cell; ACR: albumin creatinine ratio; CCR: creatinine clearance ratio; TRPC1: transient receptor potential cation channel, subfamily C, member 1.

and decreased expression of TGF-β, collagen, and fibronectin (FN) in diabetic kidney, as well as attenuated proteinuria [26], thus indicating the possibility of the approach of LNA-anti-miR-192 to the treatment of DN.

In contrast, Wang et al. found that TGF-β treatment decreased the expression of miR-192/215 in rat proximal tubular cells (NRK-52E), primary rat mesangial cells, human podocytes, and kidney of apolipoprotein E diabetic mice [27]. The discrepancies might be due to differences in cell types and animal species. It is impossible to confirm that these unconformities really exist under the same conditions. Further studies are needed to explain the differences between these results.

2.2. miR-216a and miR-217. Kato et al. dug out the miRNA-mediated link between TGF-β and Akt, which were important signaling pathways of DN in MMCs. miR-192 and TGF-β induced levels of MiR-216a and miR-217, both of which targeted PTEN, an inhibitor of Akt activation [28]. This work not only demonstrated the presence of miRNA-network regulated by miR-192/TGF-β but also, more importantly, indicated the mechanism of miRNA-mediated Akt activation by TGF-β. A further research showed that, under diabetic conditions, miR-216a was upregulated, followed by the inhibition of Y box binding protein 1 which led to increased expression of TGF-β stimulated clone 22, eventually resulting in high production of COL1α2 in MMCs [29].

Table 2: Downregulated miRNAs.

miRNAs	Targets	Biological outcome	Models	References
miR-200a/miR-141	TGF-β2	COL1, COL4, FN ↓	NRK52E cells	[54]
miR-29a/b/c	COL1, COL4	COL1, COL4 ↓	NRK52E cells, MMCs, human podocytes	[56]
miR-29a	COL4α1/2 HDAC4	COL1, COL4 ↓ Podocytes dysfunction ↓	HK-2 cells miR-29a transgenic mice Podocytes	[57] [58]
miR-29b		TGF-β/SMAD3, Sp1/NF-κB ↓	db/db mice	[4]
miR-451	YWHAZ	p38MAPK, ECM ↓	MMCs	[60]
miR-25	NOX4	NOX4 ↓	RMCs	[66]
miR-93	VEGF-A	VEGF, COL4α3, FN ↓	db/db mice, podocytes, renal microvascular endothelial cells	[67]
Let-7b	TGFBR1	SMAD3, ECM ↓	Diabetic mice (STZ), NRK52E cells	[68]

TGFBR1: transforming growth factor beta receptor 1; VEGF-A: vascular endothelial growth factor A; Sp1: specificity protein 1; HDAC4: histone deacetylase 4; YWHAZ: tyrosine 3 monooxygenase/tryptophan 5-monooxygenase activation protein, zeta; NOX4: NADPH oxidase subunit 4; NRK52E cells: rat renal proximal tubular cell line.

This study suggested a fibrosis-inducing role of miR-216a related to the pathogenesis of DN in MMCs.

2.3. miR-200b/c. miR-200b and miR-200c are among the members of miR-200 family (miR-200a, miR-200b, miR-200c, and miR-141). miR-200b/c were found downstream of miR-192, and all three of them were able to induce TGF-β1, while miR-200b/c were both increased in glomeruli from type 1 (STZ) and type 2 (db/db) mice, as well as MMCs treated with TGF-β1, suggesting an miRNA-mediated positive feedback loop of TGF-β1 autoregulation in MMCs [22]. Besides, Park et al. observed a significant increase of miR-200b/c in diabetic mouse glomeruli and TGF-β-treated MMCs. TGF-β activated Akt in MMCs by inducing miR-200b and miR-200c, both of which targeted zinc finger protein Friend of GATA 2 (FOG2), an inhibitor of PI3K activation. Importantly, miR-200b/c inhibitors abrogated the TGF-β-induced increase in protein content to cell ratio. This study suggested a new mechanism for TGF-β-induced Akt activation through FOG2 downregulation by miR-200b/c, which led to glomerular mesangial hypertrophy in the progression of DN [30].

2.4. miR-21. Except for its critical role in tumorigenesis [31–33], miR-21 is also found as a DN player. miR-21 serves as the molecular link between high glucose and PTEN and contributes to renal cell hypertrophy and matrix expansion. Overexpression of miR-21 resulted in reduction in PTEN expression and increase in Akt phosphorylation, while miR-21 sponge, a miR-21 inhibitor, reversed the DN-inducing effects of high glucose. miR-21 also inactivated proline-rich Akt substrate of 40 kDa, a negative regulator of mammalian target of rapamycin complex 1 that can mediate pathologic features of DN [23]. In line with this study, work by Zhong et al. demonstrated miR-21 as a key therapeutic target for renal injury in db/db mice. The authors found miR-21 targeted mothers against decapentaplegic homolog 7 (SMAD7), which was the repressor of TGF-β1. Importantly, transferring miR-21 knockdown plasmids into the diabetic kidneys of db/db mice ameliorated microalbuminuria, renal fibrosis, and inflammation at age 20 weeks, revealing a therapeutic potential for DN by targeting miR-21 [34].

Fiorentino et al. found that, in a mice model of type 1 diabetes, SV40 MES 13 mouse mesangial cells, as well as human kidney biopsies from patients of DN, miR-21 were significantly upregulated, which led to downregulation of tissue inhibitors of metalloproteinase 3 (TIMP3) [35]. Given that TIMP3 deficiency has emerged as a hallmark of DN [36], it is conceivable that miR-21 may be an inducer of DN. In addition, work by Wang et al. in kk-ay type 2 diabetic mice demonstrated that miR-21 contributes to renal fibrosis by downregulating matrix metallopeptidase 9/TIMP1. The ECM inducing effect of miR-21 was reversed by antagomir-21 [37]. These two studies suggested that miR-21 induces DN through regulation of TIMPs.

2.5. miR-377. miR-377 was upregulated in high glucose cultured or TGF-β treated human and mouse mesangial cells. Increased miR-377 resulted in suppression of p21-activated kinase and superoxide dismutase, which enhanced FN expression [38]. To date, this has been the only study focused on the relationship between miR-377 and DN. Interestingly, another study showed that miR-377 targeted heme oxygenase 1 (HO-1), an important antioxidant which participated in oxidative redox signaling [39]. Since HO-1

also prevents DN through antioxidative effect [40, 41], miR-377/HO-1 pathway might be a new mechanism by which miR-377 induces DN. Further studies are needed to verify the underlying mechanisms.

2.6. miR-195. Elevated expression of miR-195 was found in both STZ-induced type 1 diabetic mice and podocytes cultured in high glucose. B-cell lymphoma 2 protein levels were decreased while caspase-3 increased in podocytes after transfection with miR-195 [42]. These findings suggested that miR-195 might mediate podocyte apoptosis in DN. In line with this study, miR-195 was observed to be increased not only in STZ-induced type 1 diabetic mice but also in high glucose cultured MMCs, followed by enhanced apoptosis of MMCs [43].

Besides, miR-195 was identified as an inhibitor of sirtuin 1 (Sirt1) in DN [44]. As a histone deacetylase, Sirt1 is a key regulator which ameliorates DN via multiple mechanisms [45–48]. It is interesting to investigate the regulation of Sirt1 by miR-195 in DN and inhibiting miR-195 might be a new strategy to ameliorate DN.

2.7. miR-215. Mu et al. identified miR-215 as an epithelial-mesenchymal transition-promoting molecule in TGF-β1 treated MMCs [49]. miR-215 was found to target catenin-beta interacting protein 1, which suppressed Wnt/β-catenin signaling. Thus miR-215 activated β-catenin followed by the overexpression of alpha smooth muscle actin (α-SMA) and FN.

2.8. miR-124. Podocytes are key components of the glomerular filtration barrier and adhere tightly to glomerular basement membrane (GBM) mainly through cell-matrix adhesion receptor INTEGRIN$\alpha 3\beta 1$ [50]. Li et al. found INTEGRIN$\alpha 3\beta 1$ as a target of miR-124 [51], indicating the possible role of miR-124 in podocyte adhesion damage under mechanical stress.

2.9. miR-1207-5p. Alvarez et al. reported that a long noncoding miRNA, miR-1207-5p, was highly expressed in normal human renal proximal tubule epithelial cells, podocytes, and normal mesangial cells and was upregulated by high glucose and TGF-β1; meanwhile miR-1207-5p also increased TGF-β1, PAI-1, and FN1, all of which contributed to DN [52].

2.10. miR-135a. He et al. showed that miR-135a was markedly upregulated in serum and renal tissue from patients with DN, as well as from db/db mice, accompanied by the development of microalbuminuria and renal fibrosis. Furthermore, the authors identified transient receptor potential cation channel, subfamily C, member 1 (TRPC1), as a target of miR-135a during renal injury. Overexpression of TRPC1 was able to reverse the pathological effects of miR-135a on promoting proliferation of mesangial cells and increasing synthesis of extracellular matrix proteins. Moreover, miR-135a attenuated store depletion-induced Ca (2+) entry into cells by regulating TRPC1. Importantly, knockdown of miR-135a in diabetic kidneys restored levels of TRPC1 and reduced synthesis of fibronectin and collagen 1 *in vivo* [53]. These findings suggested that miR-135a plays an important role in renal fibrosis and inhibition of miR-135a might be an effective therapy for DN.

3. Downregulated miRNAs in DN

Several key factors are overexpressed in DN, such as TGF-β2, COL1, COL4, and NADPH oxidase subunit 4 (NOX4). These DN-inducing factors result in ECM accumulation, renal fibrosis, and oxidative stress, all of which contribute to the pathogenesis of DN. These DN-inducing factors are also targets of several miRNAs, which are downregulated in DN. It is reasonable that these downregulated miRNAs are DN-inhibiting miRNAs which lead to the decrease of these DN-inducing factors (Table 2).

3.1. miR-200a and miR-141. Although in the same family of miR-200, miR-200a and miR-141 seem to have opposite effects from miR-200b/c. In NRK52E cells, both TGF-β1 and -β2 downregulated miR-200a, which reduced expression of ECM proteins such as COL1, COL4, and FN, and so did miR-141. Interestingly, both miR-200a and miR-141 repressed TGF-β2 expression [54]. The study established a reciprocal inhibiting effect between miR-200a/miR-141 and TGF-β2. More recently, aldose reductase was found to elevate miR-200a-3p and miR-141 so as to coordinate kelch-like ECH-associated protein 1/NFE2-related factor 2, attenuating TGF-β1/2 signaling in both renal cortex of STZ-induced mice and MMCs [55].

3.2. miR-29. All three members of the miR-29 family (miR-29a/b/c) were suppressed by TGF-β1 in proximal tubular cells (NRK-52E), primary mouse mesangial cells, and human podocytes. miR-29 family repressed the expression of targeted COL1 and COL4 in both mRNA and protein levels [56]. In agreement with the study by Wang et al. [56], MiR-29a was downregulated in HK-2 cells (human proximal tubule cell line) under high glucose/TGF-β1 conditions. It directly targeted 3′UTR of COL4α1 and COL4α2, resulting in downregulation of these two fibrotic genes [57].

Study by Chen et al. demonstrated a renal-protective role of miR-29b in db/db mice, indicating that miR-29b may exert its protective effect by inhibiting TGF-β/SMAD3 signaling pathway and specificity protein 1/NF-κB-driven renal inflammation [4]. A recent finding demonstrated that hyperglycemia-induced podocyte dysfunction was ameliorated by miR-29a promotion of nephrin acetylation [58].

Different from findings aforementioned, by using a miRNA expression array, Long et al. found miR-29c as an important miRNA in inducing cell apoptosis and accumulation of ECM under diabetic environment. The authors also identified Sprouty homolog 1 as a direct target of miR-29c. Albuminuria and kidney ECM were reduced by knockdown of miR-29c with antisense oligonucleotide in db/db mice [59]. The discrepancies may be due to differences in experimental models. Further studies are required to confirm the controversial results.

3.3. miR-451. To date, only one study has shown the DN-preventing role of miR-451. Zhang et al. defined tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ), as a target of miR-451 and overexpression of miR-451 caused reduction of p38-MAPK signaling via suppression of YWHAZ [60], revealing the potential therapeutic role of miR-451 since p38-MAPK pathway was positively involved in DN [10]. However, in cancer research, a variety of studies have shown the tumor suppressing effect of miR-451. miR-451 targeted 14-3-3zeta, a phosphoserine/threonine-binding protein that inhibited nuclear accumulation of transcription factor FoxO3, a positive regulator of erythroid antioxidant genes [61]. miR-451 also inhibited cell proliferation in human hepatocellular carcinoma through direct suppression of inhibitor of kappa B kinase-beta, leading to the downregulation of NF-κB [62]. Zhang et al. also validated that miR-451 targeted CUG triplet repeat-binding protein 2, a ubiquitously expressed RNA-binding protein, known to interact with cyclooxygenase-2 (COX-2) 3′UTR and inhibit its translation [63]. Since each of FoxO3, NF-κB, and COX-2 plays a role in DN [15, 64, 65], it is possible that miR-451 may ameliorate DN through regulating these factors. Further studies are needed to verify the hypothesis.

3.4. miR-25. miR-25 level was significantly reduced both in kidneys from diabetic rats and in high glucose-treated mesangial cells, accompanied by the increases in NOX4 expression levels. An inhibitor of miR-25 effectively increased NOX4 levels. Luciferase assays showed that miR-25 directly bound to the 3′UTR of NOX4 mRNA. These data indicate that miR-25 may be a DN-protective molecule through inhibiting NOX4 [66].

3.5. miR-93. Long et al. identified vascular endothelial growth factor A (VEGF-A) as a putative target of miR-93 in kidneys of db/db mice. Cell experiments showed the forced expression of miR-93 abrogated VEGF protein secretion, while miR-93 inhibitors increased the secretion of VEGF [67].

3.6. let-7b. TGF-β1 decreased let-7b expression and induced fibrogenesis in NRK52E cells while ectopic expression of let-7b inhibited TGF-β1 receptor 1 (TGFBR1) expression, leading to reduced expression of ECM genes. Conversely, knockdown of let-7b elevated TGFBR1 expression and mimicked the profibrotic effect of TGF-β1. Importantly, let-7b expression was also reduced in kidneys of type 1 diabetic mice together with upregulated TGFBR1 [68]. Thus, let-7b seemed to show a good prospect for therapeutic intervention of renal fibrosis in DN. However, work by Schaeffer et al. [69] showed elevated let-7b under high glucose conditions, the result of which was reduced expression of transcription factor high-mobility group AT-hook 2, in turn reducing laminin subunit beta-2, which was regarded as a key component of GBM and determined glomerular barrier permeability. Further studies are needed to confirm the exact role of let-7b in DN.

4. Therapeutic Speculation of miRNAs in DN

Because of the important role of miRNAs in regulating multiple biological effects in DN, it is of great potential to develop methods to inhibit DN-inducing miRNAs or increase kidney-protective miRNAs. The aforementioned upregulated or downregulated miRNAs may be potential targets for the treatment of DN.

4.1. Silencing DN-Inducing miRNAs. There are basically four ways to silence miRNAs, including anti-miRNA oligonucleotides (AMOs), miRNA-inhibiting natural agents, miRNA sponges, and gene knockout [70]. All four methods are briefly introduced below.

4.1.1. Anti-miRNA Oligonucleotides (AMOs). AMOs are designed to complement miRNAs that are stopped from binding to their target sequences [71]. However, delivery of AMOs *in vivo* is a substantial obstacle to their effective use as therapeutics. Chemical modification of AMOs can be beneficial by improving hybridization affinity for the target mRNA, resistance to nuclease degradation, or activation of RNaseH or other proteins involved in the terminating mechanism [72]. 2′-O-Me modification as well as the 2′-O-methoxyethyl (2′-MOE) and 2′-fluoro (2′-F) chemistries is modified at the 2′ position of the sugar moiety, while LNA comprises a group of bicyclic RNA analogues in which the furanose ring in the sugar-phosphate backbone is chemically locked in an RNA mimicking N-type (C3′-endo) conformation by the introduction of a 2′-O,4′-C methylene bridge [72–76]. Among these chemical modifying methods, LNA shows the highest affinity towards complementary RNA [77, 78].

Inhibiting DN-inducing miRNAs with AMOs represented a good profile in the treatment of the disease. Transfer of miR-21 knockdown plasmids which contained LNA-anti-miR-21 into the diabetic kidneys of db/db mice at age 10 weeks significantly attenuated microalbuminuria, renal fibrosis, and inflammation at age 20 weeks [34]. Multiple low dose administration of LNA-anti-miR-192 in type 1 diabetic mice resulted in decreased miR-192 level, together with decreased COL1α2 and miR-216a/miR-217 and attenuated Akt activation [28]. In another study, injection with LNA-anti-miR-192 decreased the expression of miR-192, miR-141, miR-200b, miR-200c, COL1α2, COL4α1, and TGF-β1 in mouse renal cortical tissues [22]. Similarly, LNA-anti-miR-192 ameliorated DN in C57 type 1 diabetic mice by restoring the function of ECM inhibitor ZEB1/2, leading to downregulation of ECM genes and less albuminuria [26]. Knockdown of miR-29c by a specific antisense oligonucleotide significantly reduced albuminuria and ECM in kidneys of db/db mice [59]. miR-215 silencing *in vivo* with antagomir-215 significantly reduced miR-215-mediated β-catenin activity and decreased a-SMA and FN expression in db/db mice [49]. Antagomir-21 decreased TIMP1, COL4, and FN proteins as well as urine albumin creatinine ratio (ACR) and creatinine clearance ratio (CCR) in kk-ay mice [37]. These findings suggested a potential therapeutic prospect of AMOs in clinical use.

4.1.2. miRNA-Inhibiting Natural Agents. Some natural agents derived from food are demonstrated to have miRNA-inhibiting effect. Curcumin and its analog CDF were found to downregulate miR-21, a key miRNA in tumor aggressiveness [79]. Resveratrol also reduced prostate cancer growth and metastasis by inhibiting Akt/miR-21 pathway [80]. Because miR-21 contributes to DN, it is quite possible that curcumin and resveratrol may ameliorate DN through inhibiting miR-21 [7, 34, 36, 37].

4.1.3. miRNA Sponges. miRNA sponges contain complementary binding sites to the seed region of the miRNA of interest, which allows them to block a whole family of related miRNAs [70]. The sponges are transferred into cells by subcloning the miRNA binding site region into a vector containing a U6 small nuclear RNA promoter with 50 and 30 stem-loop elements [81].

A study using miR-21 sponge effectively inhibited endogenous miR-21 at the cellular level and prevented downregulation of PTEN and phosphorylation of Akt induced by high glucose in rat and human mesangial cells [23]. However, the application of miRNA sponges in DN animal models has not been reported, although it has already been used in animal models of cardiac hypertrophy, immune response, and breast cancer [82–84]. Studies in animal models are needed to observe the utility of this method towards DN.

4.1.4. Genetic Knockout. Gene-knockout mice lose the whole function of miRNAs. The knockouts can be either a miRNA itself or key miRNA processing factors such as Drosha, Dicer, and argonaute 2 (Ago2). Mice with podocyte-specific deletion of Dicer induced proteinuria and glomerulosclerosis [85]. Another study showed that Dicer-knockout in podocytes led to rapid glomerular and tubular injury [86].

On the other hand, a specific miRNA knockout showed a promising effect on the prevention of DN. miR-192-knockout mice were protected from key features of DN [87] and miR-21-knockout mice suffered far less interstitial fibrosis in response to kidney injury [88].

4.2. Restoring Renal-Protective miRNAs. By using miRNA mimics, miRNA expression vectors, miRNA-containing exosomes, and miRNA-inducing natural agents, levels of renal-protective miRNAs can be restored and thus lead to the protection from DN.

4.2.1. miRNA Mimics. miRNA mimics are double-stranded synthetic miRNA oligonucleotides. The guide strand is identical to the mature miRNA sequence, while the other strand called passenger strand is partially or fully complementary to the guide strand [89]. When transfected into cells, the guide strand which mimics the function of certain miRNA regulates protein-coding genes in a miRNA-like manner [90]. miRNA oligo mimics are easy to synthesize and introduce into cells using lipid reagents or electroporation and are easily achievable in most cellular situations. However, high cost for synthesis and purification and rapid clearance following transfection are the disadvantages. A variety of miRNA mimics showed miRNA mimicking effects on cancers both *in vitro* and *in vivo* [91–94]. However, there is no study focused on miRNA mimics in DN. Thus, replacement of renal-protective miRNAs would be a new strategy for the attempt to the treatment of DN.

4.2.2. miRNA Expression Vectors. miRNA expression vectors are engineered to express miRNAs of interest. In a plasmid or viral vector, a certain miRNA can be expressed by a short hairpin RNA (shRNA) using polymerase II or III promoter. The shRNA is processed into mature miRNA by Dicer before loading into RISC [95]. Artificial miRNA vector which contained natural miRNA precursor inhibited the expression of target mRNA [96]. The knockdown effect of shRNAs on the expression of target gene was striking and stable [74]; however, it might saturate the Exportin 5 pathway of endogenous miRNAs, leading to off-target effects with fatal consequences [97]. miRNA expression vectors have the advantages of longer expression and reduced likelihood of off-target effects because the guide and passenger strands are completely natural to the cell [89]. The limitations in clinical applications include possible insertion of genetic material into the specific location of the genomes of the host cells and causing possible immune responses. To date, no studies utilizing miRNA vectors are found on DN.

4.2.3. miRNA-Containing Exosomes. Exosomes are 40–100 nm membrane vesicles which contain proteins, mRNAs, miRNAs, or signaling molecules and are secreted into the extracellular space by numerous cell types [98]. Valadi et al. demonstrated that exosomes transferred miRNAs from their cell of origin to target cells [99]. In addition to miRNAs, pre-miRNA could be identified in mesenchymal stem cell-derived exosomes [100]. Therefore, the miRNAs-transferring ability of exosomes offers the promise that they may be used for therapeutic purposes for DN. Exosomal miRNAs have been discovered as diagnostic biomarkers of DN [101, 102]. However, studies on exosomal miRNAs in preventing or ameliorating DN are still wanted in the future.

4.2.4. miRNA-Inducing Natural Agents. Difluorinated curcumin (CDF), a curcumin analog, increased the expression of miR-200a in pancreatic cancer cells [103]. Isoflavone and 3,3′-diindolylmethane (DIM) restored the expression of let7-b and led to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells [104]. Because of the renal-protective role of miR-200a [54, 105] and let7-b [68], CDF, isoflavone, and DIM might prevent DN through regulating these two miRNAs.

To date, there has not been a successful clinical intervention of miRNAs towards diseases. However, several miRNA interventions have been in different clinical developmental stages. miR-122 inhibitor against hepatitis C virus infection is in phase II clinical trial [106]. miR-34 mimic against liver cancer or metastasized cancer involving liver is in phase I clinical trial [90]. let-7 mimic against cancer (details undisclosed) is in preclinical stage [107]. Inhibitors of miR-21, miR-208, miR-195, miR-221, miR-103/105, and miR-10b are in preclinical

stage against cancer/fibrosis, heart failure/cardiometabolic disease, postmyocardial infarction remodeling, hepatocellular carcinoma, insulin resistance, and glioblastoma, respectively [107]. It is noted that, among these miRNAs, miR-21 and miR-195, let-7 are all DN-related, which might provide hope for the treatment of DN.

5. Conclusions

In summary, several miRNAs are related to DN. Some of them take part in the pathogenesis and development of the disease while others serve as DN-killers or -preventers. Therefore, it would be wise to elevate the renal-protective miRNAs and reduce DN-inducing ones.

miRNAs established a vast ocean for researchers to dive into and find the pathogenesis of disease and the potential target for therapeutic intervention. The prospect of miRNA-based intervention is bright. However, there are still challenges. For example, the exact and detailed regulation and function of miRNAs are still not fully understood. A certain miRNA may have several target genes. Thus, either upregulation or downregulation of a miRNA would have multifactorial effects, including the expected effect as well as side effects. Experimental verification of target genes also seems to be hard work, for the miRNA regulations are basically at a translation level. Better understanding of miRNA biogenesis and function will be beneficial for better application of miRNA-based treatment for diseases, including DN.

Abbreviations

ACR: Albumin creatinine ratio
AMOs: Anti-miRNA oligonucleotides
α-SMA: Alpha smooth muscle actin
BCL2: B-cell CLL/lymphoma 2
CCR: Creatinine clearance ratio
Col: Collagen
DN: Diabetic nephropathy
ECM: Extracellular matrix
ERK: Extracellular signal-regulated kinases
FN: Fibronectin
FOG: Friend of GATA
HDAC4: Histone deacetylase 4
HMC: Human mesangial cell
MAPK: Mitogen-activated protein kinase
miRNA: MicroRNA
MMC: Mouse mesangial cell
mTORC1: Mechanistic target of rapamycin complex 1
NF-κB: Nuclear factor kappa B
NOX4: NADPH oxidase subunit 4
NRK52E: Rat renal proximal tubular cell line
PAI-1: Plasminogen activator inhibitor-1
PAK1: p21 activated kinase
PI3K-Akt: Phosphoinositide 3-kinase-protein kinase B
PRAS40: Proline-rich Akt substrate 40
RISC: RNA-induced silencing complex
RMC: Rat mesangial cell
SOD: Superoxide dismutase
Sp1: Specificity protein 1
SPRY1: Sprouty homolog 1
STZ: Streptozotocin
TIMP: Tissue inhibitors of metalloproteinase
TGF-β: Transforming growth factor-β
TGFBR1: Transforming growth factor beta receptor 1
TRPC1: Transient receptor potential cation channel, subfamily C, member 1
VEGF-A: Vascular endothelial growth factor A
YBX1: Y box binding protein 1
YWHAZ: Tyrosine 3 monooxygenase/tryptophan 5-monooxygenase activation protein, zeta
ZEB1/2: Zinc finger E-box binding homeobox 1/2.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Hao Wu collected information and accomplished the paper. Lili Kong drew the figures and tables. Shanshan Zhou, Wenpeng Cui, Feng Xu, Manyu Luo, and Xiangqi Li did the editing work. Yi Tan and Lining Miao provided the financial support, gave advice for the whole work, and made the decision to submit the paper for publication.

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Review Article

Histone Lysine Methylation in Diabetic Nephropathy

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Diabetic nephropathy (DN) belongs to debilitating microvascular complications of diabetes and is the leading cause of end-stage renal diseases worldwide. Furthermore, outcomes from the DCCT/EDIC study showed that DN often persists and progresses despite intensive glucose control in many diabetes patients, possibly as a result of prior episode of hyperglycemia, which is called "metabolic memory." The underlying mechanisms responsible for the development and progression of DN remain poorly understood. Activation of multiple signaling pathways and key transcription factors can lead to aberrant expression of DN-related pathologic genes in target renal cells. Increasing evidence suggests that epigenetic mechanisms in chromatin such as DNA methylation, histone acetylation, and methylation can influence the pathophysiology of DN and metabolic memory. Exciting researches from cell culture and experimental animals have shown that key histone methylation patterns and the related histone methyltransferases and histone demethylases can play important roles in the regulation of inflammatory and profibrotic genes in renal cells under diabetic conditions. Because histone methylation is dynamic and potentially reversible, it can provide a window of opportunity for the development of much-needed novel therapeutic potential for DN in the future. In this minireview, we discuss recent advances in the field of histone methylation and its roles in the pathogenesis and progression of DN.

1. Introduction

Diabetic nephropathy (DN) is a well-known microvascular complication of diabetes and the leading cause of end-stage renal disease (ESRD) all over the world which contributes greatly to morbidity, mortality, and most health care costs [1]. DN clinically manifests as initial increase in glomerular filtration (GFR), microalbuminuria, proteinuria, glomerulosclerosis, increased creatinine levels, and eventual decreased GFR [2–4]. Well-described characteristic histological and pathological changes of DN in glomerulus, tubulointerstitium, and vasculature are virtually indistinguishable in both type 1 and type 2 diabetes [5, 6]. Typical glomerular changes include mesangial cell proliferation and hypertrophy caused by excessive extracellular matrix (ECM) protein accumulation and glomerular basement membrane (GBM) thickness, podocyte loss, and foot process effacement, which eventually lead to nodular glomerular sclerosis known as Kimmelstiel-Wilson lesions [7–9]. Similar changes occur in the tubulointerstitium after glomerular changes, including tubular basement membrane (TBM) thickness, tubular hypertrophy, and interstitial fibrosis due to EMT process [10, 11]. Hyaline arteriolosclerosis is often prominent caused by endothelial dysfunction and inflammation [5, 9, 12].

All the cell types of kidney were involved in the pathogenesis of DN including mesangial cells (MC), endothelial cells (EC), glomerular podocytes, tubular epithelia, interstitial fibroblasts, vascular endothelia, and infiltrating monocytes/macrophages/lymphocytes due to hyperglycemia. Hyperglycemia and complex interactions between environmental and genetic factors are responsible for the development of DN [5, 13], which lead to a lot of intracellular events including increased flux of polyols and hexosamines, activation of reactive oxygen species (ROS), and advanced glycation end products (AGEs); activation of the PKC, renin-angiotensin system (RAS), transforming growth factor β-Smad-mitogen-activated protein kinase (TGF-β-Smad-MAPK), Janus kinase-signal transducer and activator of

transcription (JAK-STAT) pathways, and G protein signaling; deregulated expression of cyclin kinases, and their inhibitors; and aberrant expression of ECM proteins, ECM-degrading enzymes, metalloproteinases, and their inhibitors [5, 14–23]. All the above factors can induce aberrant expression of profibrotic and proinflammatory cytokines, cell-cycle genes, and ECM genes involved in DN [24]. There is cross talk among the above different signaling pathways that can amplify the aberrant pathogenetic gene expression and lead to the progression of DN. Emerging evidences showed that microRNAs (miRNAs) are involved in the cross talk among RAS, AGE/RAGE, and ROS in the context of DN [25]. Despite current understanding of the mechanism of DN, still there are not enough therapeutic approaches in preventing the progression of DN to ESRD, suggesting that further mechanism and mediators should be investigated for DN.

Another potential reason for the long-term progression of diabetic complication in kidney could be a metabolic memory phenomenon, early exposure of the target cells to high glucose (HG), leading to persistence of its deleterious effects after effective glycemic control. This cellular memory phenomenon was revealed by large-scale multicenter clinical trials such as the Diabetes Control and Complications Trial (DCCT) and the follow-up observational Epidemiology of Diabetes Intervention and Complications (EDIC) of the same cohort examined the long-term benefits of intensive therapy following the blood glucose normalization in type 1 diabetes group as well as in experimental models, and in type 2 diabetes patients the similar phenomenon referred to as legacy effect from the large-scale United Kingdom Prospective Diabetes Study (UKPDS) was also identified. The consistency across these studies is striking: that early glycemic control significantly delays but does not totally block the onset and the progression of diabetic nephropathy in both type 1 and type 2 diabetes [26–28]. Similar results were reported in recent animal models (diabetic dogs or rats) [29–31] and cell culture models (vascular smooth muscle cells and ECs) [32, 33], but the molecular determinants of metabolic memory in renal cells remain poorly understood. Since metabolic memory is bad for the prevention and treatment of DN, exploring the mechanisms underlying metabolic memory seems important. In recent years, epigenetic regulatory mechanisms have been well studied. Waddington originally defined epigenetics mainly to describe changes during embryonic development as "the casual interactions between genes and their products which bring the phenotype into being" [34]. More recently it has been broaden as "the structural adaptation of chromosomal regions so as to register, signal, or perpetuate altered activity states" to explain alterations in the chromatin state and structure in response to various cues [9]. Gene regulation controlled by the epigenetic mechanisms can play key roles in various health problems (type 2 diabetes, metabolic memory, autoimmune diseases, cancer, and autism) and also in phenotypic differences between monozygotic twins [35].

Epigenetics is widely known as the regulation of gene expression without changes in DNA sequence, and epigenetic modification consists of classic epigenetic mark as DNA methylation and posttranslational modifications (PTMs) of histones. These traditional epigenetic mechanisms, along with noncoding RNA (ncRNA), can modulate gene expression in the cell type specific pattern [9, 36]. Changes in epigenetic states can cause gene deregulation and pathological outcomes and can be implicated in the pathogenesis of various kinds of diseases including DN, uncovering how environmental factors, diets, and physical activities influencing the epigenetic modification could provide new insights into the pathogenesis of DN and new diagnostic biomarkers as well as preventive and therapeutic approaches for early intervention [9]. In eukaryotes, the way to achieve the regulation of genetic information mentioned above should be via the package of chromatic DNA which is inside a higher-order structure made up of a series of subunits named nucleosome. Every nucleosome has the folding of 147-bp liner DNA (approximately 2 meters) around the octamers comprising two copies of core histone proteins H2A, H2B, H3, and H4 [37, 38]. Histone tails can undergo more than 60 different types of modification; dynamic chromatin structure is modulated by PTMs on histones including acetylation and methylation of lysine (K) and arginine (R), phosphorylation of serine (S) and threonine (T), ubiquitylation and sumoylation of lysines, and ribosylation [39, 40]. It is difficult to decode the specific PTMs at the level of single histones and single nucleosomes; mounting evidence suggests that histone modifications "communicate" and influence each other [38]. Recent advances in the high-throughput technologies have greatly helped in cataloguing the proteins modified by abovementioned PTMs [41]. In the recent 14 years research on histone methylation has been the most flourishing field of epigenetics [42]; it is usually considered a prevalent modification among core histone tails and is one of the most stable PTMs and could be key factors in diabetic complications, but few studies have been done in DN. Here, we summarize this emerging area of research related to abnormal epigenetic interactions in DN, particularly focusing on the histone lysine methylations and their roles in the development and progression of DN.

2. Histone Methylations in DN

Methylation of a lysine residue was first reported in the *flagellin* protein of *Salmonella typhimurium* in 1959 by Ambler and Rees [41, 48] and histone methylation was depicted for the first time in 1964 [49]. Histone can be mono-, di-, or trimethylated on lysine and arginine residues which can add another layer of complexity to the posttranslational status on the histone tails; the predominant methylated sites in histones H3 include lysines 4, 9, 27, 36, and 79, while lysine 20 is methylated in H4 [50]. Histone methylation is associated with gene repression or activation depending on which residue is modified; in general, methylation at H3K4, H3K36, and H3K79 correlates with gene transcription, while methylation at H3K9, H3K27, and H4K20 correlates with transcriptional repression [51]. Unlike the histone acetylation linked to an "open" chromatin state and to the activation of gene expression, histone methylations have more diverse results: they can be marks of the active, "poised," and repressive states of chromatin that has resulted in more attention in

the explosion of studies to investigate the biological and pathological functions in the prevalent diseases including DM and its consequent complications such as DN. Below, we focus on the less well-studied role of histone lysine methylation in the development and progression of DN by two respective fields: the active chromatin marks and the repressive chromatin marks.

2.1. Active Chromatin Marks. In general, H3K4me1/2/3, H3K36me2/3, and H3K79me2 are associated with transcriptionally active regions; genome-wide mapping of above histone methylation regions showed important roles in the islet-specific promoters and enhancers for the pathogenesis of diabetes [9, 52]. Increasing evidences suggest that histone methylation are involved in the regulation of extracellular matrix (ECM) and inflammatory genes in almost all kinds of renal cell types associated with the pathogenesis of DN; we will talk about the functions of H3Kme in DN in the order of cell culture model, animal model, and DN patients.

2.1.1. H3K4 Methylation. Recent studies indicate that H3K4me1/2/3 are important for the deregulation of key genes in the pathogenesis of DN. Among lots of factors and signaling contributing to DN pathogenesis, TGF-β can play important role in the expression of fibrotic and ECM genes such as collagen 1α1 (*Col1α1*), plasminogen activator inhibitor 1 (*PAI-1*), and connective tissue growth factor (*CTGF*) in renal cells. A recent study examined histone H3K4me in rat mesangial cell (RMC) treated with HG and TGF-β. TGF-β can induce *Col1α1*, *PAI-1*,and *CTGF* gene upregulation, which was associated with increased levels of H3K4me marks (H3K4me1/2/3) activation at their respective promoters [43]. HG treatment on RMCs can lead to similar changes in histone H3K4me at ECM associated genes promoters also, but the effects on the H3Kme changes by HG can be blocked by a TGF-β specific antibody, which showed a key role of TGF-β in the HG-induced histone H3K4me [43].

In transient hyperglycemic cell culture models, endothelial cells were stimulated by HG for 16 hours and then remained in normal glucose concentration medium, pleiotropic transcription factor *NF-κB* subunit *P65* displayed sustained activation, and level of H3K4me1, but not H3K4me2 or H3K4me3, in the proximal promoter region of the *p65* gene was increased [33, 53]; two *NF-κB p65*-activated inflammatory genes *MCP-1* and *VCAM-1* were increased and remained elevated, and transient hyperglycemia-induced *p65* gene upregulation was prevented by overexpression of *UCP-1*, *MnSOD*, or *GLO1*. Aortic endothelial cells isolated from nondiabetic mice model show that transient hyperglycemia can induce increased H3K4me1 at the *p65* promoter and increase *p65* gene transcription [33]. These results prove that metabolic memory exists in the vascular dysfunction arising from hyperglycemic exposure due to H3K4me modification.

Evidences of infiltrating blood cells recruitment are dependent on the key proinflammatory pathways in diabetes and vascular complications [54]; in cultured human monocytes (THP-1) stimulated with high glucose, results from genome-wide location analyses with ChIP coupled with DNA microarrays (ChIP-on-chip) show that H3K4me2 are dynamically changed; similar changes can be seen in the monocytes from type 1 and type 2 diabetes, which can provide clues to the "metabolic memory" phenomenon [55] by such profiling approaches.

Evidence for histone methylation changes in DN also comes from studies in animal models. One report showed that in uninephrectomized db/db mice kidney H3K4me2 level was increased in accordance with glomerular cell proliferation, albuminuria, and glomerular rate (GFR) reduction, and *MCP-1/CCL2* antagonist treatment can prevent DN histopathological damage and H3K4me2 change in uninephrectomized db/db mice [56]. Results of a recent study from type 2 diabetes model db/db mice showed that, relative to db/+ mice, H3K4me1 level in glomeruli from db/db mice was increased in accordance with RNA polymerase II recruitment enhancement at the promoters of PAI-1 and RAGE (receptor for advanced glycation end products), whereas no differences were noted for H3K4me2/3. Emerging evidences have implicated that Losartan, an Ang II type 1-receptor blocker (ARB), can slow down the progression of DN. Losartan treatment for 10 weeks in db/db mice can ameliorate key factors of DN, and there was no change in the H3K4me1 recruitment at *PAI-1* and *RAGE* promoters, suggesting that it cannot reverse H3K4me changes observed in the db/db mice and accounting for the incomplete inhibitory effect of ARBs in DN patients [44]. Another study in db/db mice kidney showed that ER stress can trigger the expression of inflammatory gene *MCP-1* associated with increased H3K4me1 at *MCP-1* promoter mediated by XBPs-induced *SET7/9* elevation [57].

Another report demonstrated that in type 1 diabetes models OVE26 mice and STZ induced rat renal gene expression of *Cox2*, *S100A4/FSP1*, and vimentin in both, *MCP-1* only in mice was upregulated, associated with increased H3K4me2 levels [47]. Some other studies showed that H3K4me3 at proinflammatory genes (*MCP-1* and *TNF-α*), profibrotic genes (*TGF-β1* and *collagen III*), and histone-modifying enzyme (Set1 and BRG1) were increased in renal ischemia-reperfusion injury animal models [58, 59], which provide hints for the DN underlying mechanism study in the future. All these results from animal models have shown that histone methylation plays important roles in the progression of DN in fibrotic, inflammatory, and oxidant stress ways.

2.1.2. H3K36 Methylation. H3K36me3 was a chromatin mark associated with transcriptional elongation [60, 61]. In the study of glomeruli from db/dbH_2O mice compared with db/+H_2O mice, levels of H3K36me3 were higher at *MCP-1* and *RAGE* loci, and similar result was seen at the *PAI-1* gene but shows no statistical difference, and Losartan treatment for 10 weeks can slightly decrease H3K36me3 levels at the *RAGE* and *PAI-1* loci but not at the *MCP-1* gene [44]. This can further imply the roles of H3K36me in the progression of DN.

2.1.3. H3K79 Methylation. Unlike most of the methylated sites that are located in the histone H3 tail, H3K79 methylated

site is located in the histone globular domain. Methylation of H3K79 is catalyzed by the disruptor of telomeric silencing proteins DOT1/DOT1L and plays an essential role in cell cycle regulation, embryonic development, DNA damage response, hematopoiesis, cardiac function, and the development of leukemia [51]. One report showed that dynamic regulation of H3K79me was involved in fluid reabsorption essential for blood pressure control and electrolyte homeostasis in kidney collecting ducts: decreased H3K79me at epithelial sodium channel promoter can lead to increased gene expression in response to aldosterone signaling since H3K79 hypermethylation was associated with gene repression [62–64]. A recent study shows that both mice model and DN patients can develop polyuria due to the upregulation of Aqp5, in which Aqp5 acted as an Aqp2 binding partner and regulator and can impair Aqp2 membrane localization, and decreased H3K79me2 may contribute to the changes in DN patients and in mouse cortical collecting duct M1 cells models [45]. The known results can lead to great interest in investigating how specific gene promoters H3K79me were altered in other renal cells under diabetic conditions.

2.2. Repressive Chromatin Marks. H3K9me2/3, H3K27me3, and H4K20me3 are generally associated with gene silence or repression, and we will talk about them one by one following the orders of cell culture model, animal model, and DN patients mentioned above.

2.2.1. H3K9 Methylation. Several recent reports showed that histone methylation may be responsible for the "metabolic memory" phenomenon leading to long-term changes in diabetic complications including DN. High glucose can stimulate the decreased H3K9me3 levels at the promoters of key inflammatory genes (*IL-6*, *MCSF*, and *MCP-1*) associated with increased inflammatory genes expression in normal human vascular smooth muscle cells (VSMC), similar chromatin lysine methylation changes were demonstrated in db/db mice VSMC compared to nondiabetic control db/+ VSMC, and TNF-α induction can lead to sustained decreases of H3K9me3 at promoters in accordance with increased inflammatory genes expressions in db/db VSMC [46, 63].

In TGF-β and HG treated RMCs models, TGF-β and HG can result in similar changes: induced *Col1α1*, *PAI-1*, and *CTGF* genes upregulation were associated with reduced levels of repressive marks H3K9me2 and H3K9me3 at their respective promoters; TGF-β specific antibody can block high glucose-induced H3K9me reduction at promoters similar to the blockade of H3K4me levels mentioned above [43]. Another study result showed that short time high glucose stimuli can induce a sustained reduction of H3K9me2 and H3K9me3 level on the *NF-κB p65* promoter even after glucose concentration returned to normal, suggesting that changes in histone methylation were associated with and potentially could partly explain the phenomenon of "metabolic memory" [53]. Dynamic change in H3K9me2 can be seen in both cultured monocytes (THP-1) treated with high glucose and isolated monocytes from diabetic patients with genome-wide location analyses with ChIP coupled with DNA microarrays (ChIP-on-chip) [55] and in lymphocytes from type 1 diabetic patients H3K9me2 levels linked to immune and inflammatory pathways associated with type 1 diabetes, and its complications including DN in a subset of genes are increased with ChIP-on-chip study versus healthy controls, suggesting that histone methylation is cell type specific and relatively stable regardless of age or gender [65], which open an access to understanding of the pathogenesis for the progression of DN [66].

There are also evidences of H3K9me changes in DN from db/db mice glomeruli. ChIP assays results show that H3K9me2 and H3K9me3 levels in db/dbH_2O mice at both *PAI-1* and *RAGE* gene promoters were lower compared with db/+H_2O group, which was inversely correlated with their upregulation, and Losartan treatment cannot reverse DN-related changes but has further lower levels of H3K9me2 and H3K9me3 at *PAI-1* and *RAGE* gene promoters [44]. All the results from experimental models supplement roles of H3K9me in the development of DN.

2.2.2. H3K27 Methylation. H3K27me is a "mark" associated with gene repression [67, 68]. A study result of ChIP assays from type 2 diabetes animal model db/dbH_2O mice shows that H3K27me3 levels at *RAGE* and *PAI-1* promoters were decreased compared with db/+H_2O, and Losartan treatment had little or no effect on DN-related H3K27me3 levels at the above two genes promoters [44]. Another report showed that in OVE26 mice and STZ induced rat type 1 diabetes models renal gene expressions of *Cox2* and *MCP-1* only in mice were upregulated, associated with decreased H3K27me3 levels, which was accompanied by H3K27me3 demethylase KDM6A species-specific increases in mice but not in rats [47]. Overall results of H3K27me3 further imply the roles of histone methylation in DN.

2.2.3. H4K20 Methylation. Methylation of histone H4 was firstly discovered almost half a century ago and the catalyzing enzymes were identified recently. Histone H4 methylation was mostly detected on lysine 20; H4K20me1 and H4K20me2 are involved in DNA replication and damage repair, whereas H4K20me3 is related with gene repression; H4K20me1 was only catalyzed by SET8 (also known as PR-SET7), where H4K20me2 and H4K20me3 were predominantly mediated by SUV4-20H1 and SUV4-20H2 [50]. In a STZ induced rat model, poor glucose control can lead to retinal key antioxidant gene mitochondrial superoxide dismutase (*SOD*) downregulation by increased promoter levels of H4K20me3 through increased corresponding methyltransferase SUV4-20H2 recruitment to *SOD* gene promoter, suggesting that diabetic retinopathy is related with *SOD* repression in retinal ECs [29]. Much more interests should be of in the study of H4K20me in the pathogenesis of DN.

3. HMTs and HDMs in DN

Histone methylation is generally considered to be relatively more stable and is mediated by histone methyltransferases (HMTs) and histone demethylases (HDMs), which increase

the complexity of the histone methylation in the pathogenesis of diseases and diabetic complications. Usually histone H3 lysine 4 methylation (H3K4me) can be mediated by lots of HMTs such as SET1/COMPASS, MLL1-4 (mixed lineage leukemia 1–4), SMYD2/3 (SET and MYND domain 2/3), and SET7/9 [69–74] and is associated with gene activation. On the other hand, histone H3 lysine 9 methylation (H3K9me) can be mediated by SUV39H1/2 (suppressor of variegation 3–9 homolog 1/2), G9a, GLP (G9a-like protein), SETDB1/ESET (SET domain, bifurcated 1/ERG-associated protein with SET domain), and Eu-HMTase1 [69, 71, 72, 75, 76] and is associated with gene repression. In addition to these, there are several lysines, including histone H3 lysine 27 methylation (H3K27me) mediated by EZH2 [77], histone H3 lysine 36 methylation (H3K36me) mediated by Set2 [78], and histone H3 lysine 79 methylation (H3K79me) mediated by Dot1 [51] that can be methylated to various degrees leading to altered gene expression. Now it is known that even lysine methylation is one of the most stable epigenetic modifications; it can be reversible by histone demethylase. The first identified histone demethylase is lysine demethylase 1 (LSD1), which can specifically remove H3K4me and H3K9me marks [79, 80]. Recently, a lot of lysine demethylases have been identified with varying specificities for different histone lysine residues [71, 81–83] whose nomenclature has been changed to lysine demethylases (KDMs). Dynamic regulation of lysine methylation can play important roles in various diseases and can be key factors in diabetic complications including DN.

Suv39h1 is a known HMT mediating H3K9me3; its protein level was decreased, associated with increased inflammatory genes expression and decreased H3K9me3 levels at inflammatory gene promoters in db/db mice VSMC compared to db/+ VSMC; Suv39h1 overexpression in db/db VSMC can reverse partial diabetic phenotype; TNF-α can induce inflammatory genes expression with corresponding decreased Suv39h1 occupancy and H3K9me3 levels at promoters [46, 63], suggesting a new direction for demonstrating potential roles of chromatin histone methylation in DN.

HMT SET7/9 recruitment and H3K4me marks appear to be characteristic of the insulin gene promoter activation only in cells associated with insulin production such as β cells, non-β cells, and embryonic stem cells (ES) [63, 84, 85]. Another study in monocyte showed that SET7/9 knockdown can decrease H3K4me1 at *MCP-1* and *TNF-α* promoters with a corresponding decreased NF-κB subunit occupancies at promoters, which suggest that knockdown of SET7/9 can attenuate TNF-α induced key inflammatory genes expression in an *NF-κB*-dependent manner. To further verify the effect of histone methylation and HMTs/HDMs in "metabolic memory," results from recent cell culture studies show that transient hyperglycemia can induce the increased recruitment of the identified histone methyltransferases (HMTs) SET7/9 and histone demethylase (HDMs) LSD1 to the *p65* promoter in aortic endothelial cells [33, 53], which can lead to better understanding of the basis and strategies to reduce the burden of diabetic complications such as DN.

Recent studies have shown that TGF-β stimulation in rat renal mesangial cells can increase SET7/9 gene expression and SET7/9 recruitment to the promoters of key fibrotic genes (including *PAI-1* and *CTGF*) linked to DN, which are associated with active H3K4me1 occupancy; high glucose stimulation can lead to similar change in RMCs; TGF-β specific antibody treatment can reverse HG induced gene expression and promoter histone methylation changes; these results highlight a key role of histone methylation and HMT SET7/9 in modulating renal gene expression leading to the pathogenesis of DN [43].

A recent study of QPCR arrays screen of 86 genes encoding epigenetic-modifying enzymes and followed by RT-qPCR validation in the glomeruli shows that H3K4 methyltransferases (Setd4 and Setd7), H3K36me3 methyltransferase (Setd2), and H3K9me3 demethylases (Jmjd2 family) were increased in db/dbH_2O mice, which can be inhibited by Losartan treatment [44]. A study mentioned above shows that renal Aquaporin 2- (*Aqp2-*) expressing cells (DOT1L^{AC}) deficient mice can develop polyuria due to the upregulation of *Aqp5*, and decreased H3K79me2 caused by DOT1L deficiency may contribute to the changes in mouse cortical collecting duct M1 cells models [45]. Evidences are progressed in investigating roles of HMTs/HDMs in the pathogenesis of DN.

Overall, these results of HMTs, HDMs, and interactions between them in various experimental models have shown the respective roles in cells relevant to DN but not sufficient, and it is anticipated that further research in the field of HMTs and HDMs may lead to clear description of the pathogenesis of DN.

4. Conclusions and Perspectives

Experimental studies including cell and animal models as well as clinical studies have clearly revealed deleterious results of hyperglycemia and importance of good glucose control in preventing the onset or progression of DN, and less proper therapeutic approaches are demonstrated. Histone lysine methylation is a dynamic process which enables another layer of gene expression control so that genes can be turned off in a cell type specific manner in response to various signaling and environmental stimuli, which has been found to play important roles in gene deregulation associated with various diseases [86]. We summarized ever demonstrated histone lysine methylation results in DN experimental models and fewer results in DN patients, which can partly explain the pathogenesis of DN but are not sufficient; new more critical information for DN will be provided with the development of microarrays and massively parallel NGS platforms. We also list histone lysine methylation changes and sustained corresponding inflammatory genes expression in endothelial cells under transient high glucose condition [33]; histone lysine methylation has similar effect in VSMC derived aortas of db/db mice, which provide new insights into metabolic memory; thus, it is likely that similar histone methylation changes can also occur in cells such as renal mesangial cells, tubules, and podocytes that are involved in DN. The interaction between HMTs and HDMs mentioned above adds complexity in DN gene expressions through histone lysine methylations mechanism (Table 1) but is not sufficient to

Table 1: Histone lysine methylations and HMTs/HDMs in DN.

Lysine	State	HMTs	HDMs	Target renal loci	Effects in DN	References
H3K4	me1	SET7/9	LSD1	RMCs, ECs, and mice glomeruli	Upregulate profibrotic gene and stimulate NF-κB activated inflammatory genes	Sun et al. [43]
	me2	MLL1-4	LSD1	RMCs		El-Osta et al. [33]
	me3	MLL1-4		RMCs		Reddy et al. [44]
H3K36	me3			Mice glomeruli	Stimulate profibrotic and proinflammatory genes expression	Reddy et al. [44]
H3K79		DOT1/DOT1L		Kidney collecting ducts	Develop polyuria	Wu et al. [45]
H3K9	me2	SUV39H1/2	LSD1	RMCs, monocyte/lymphocyte, and mice glomeruli	Repress inflammatory/profibrotic gene expression	Sun et al. [43]
		G9a				Villeneuve et al. [46]
	me3	SUV39H1		Human/mice VSMCs, RMCs, and mice glomeruli		Reddy et al. [44]
H3K27	me3	EZH2	KDM6A	Mice glomeruli	Repress profibrotic genes expression	Reddy et al. [44]
						Komers et al. [47]
H4K20	me1	SET8			Repress SOD expression	Zhong and Kowluru [29]
	me2	SUV4-20H1/2				
	me3	SUV4-20H1/2				

imply the whole pathogenesis of DN. Like HDACs inhibitors have been shown to be a novel class of therapeutic agents in diabetic kidney injury [87–89]; evidences showed that inhibitors of various HMTs could be new epigenetic therapy agents for cancers [77, 90], which is hoped to be a new therapeutic field for DN in the future. In addition to histone lysine methylation studies, advances in other mechanisms such as microRNA mediated mechanism need to be explored for preventing the progression of DN.

Conflict of Interests

The authors have no conflict of interests to declare.

Acknowledgments

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Research Article

Peripheral Neuropathy and Tear Film Dysfunction in Type 1 Diabetes Mellitus

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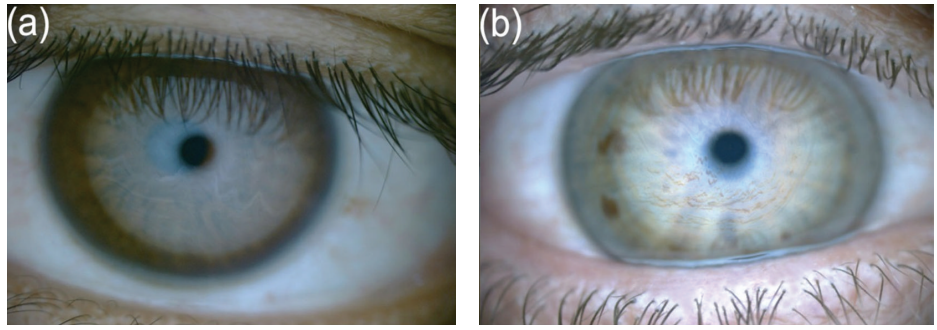
Purpose. To compare tear film metrics in patients with type 1 diabetes mellitus (DM) and healthy controls and investigate the association between peripheral neuropathy and ocular surface quality. *Methods.* Dry eye symptoms were quantified in 53 patients with type 1 DM and 40 age-matched controls. Ocular examination included tear film lipid layer thickness grading, tear film stability and quantity measurement, and retinal photography. DM individuals additionally underwent a detailed neuropathy assessment. *Results.* Neither mean age nor dry eye symptom scores differed significantly between the DM and control groups ($P = 0.12$ and $P = 0.33$, resp.). Tear lipid thickness ($P = 0.02$), stability ($P < 0.0001$), and quantity ($P = 0.01$) were significantly lower in the DM group. Corneal sensitivity was also reduced in the DM group ($P < 0.001$) and tear film stability was inversely associated with total neuropathy score ($r = -0.29$, $P = 0.03$). *Conclusion.* The DM group exhibited significantly reduced tear film stability, secretion, and lipid layer quality relative to the age-matched control group. The negative correlation between tear film parameters and total neuropathy score suggests that ocular surface abnormalities occur in parallel with diabetic peripheral neuropathy.

1. Introduction

While cataract and retinopathy have been extensively researched in patients with diabetes mellitus (DM), only a fraction of the published research has been dedicated to ocular surface complications. However, dry eye symptoms and signs of epithelial fragility, punctate keratopathy, persistent epithelial defects, and decreased corneal sensitivity are not uncommon in DM [1–3]. Compromised innervation of the cornea in patients with DM has also been described [4–6]. Tear film dysfunction, characterised by impairment in tear quantity and quality, can occur in association with abnormal corneal innervation due to the intimate, functional relationship between the cornea and the preocular tear film [7, 8]. The resulting dry eye is a recognised cause of debilitating, chronic ocular irritation symptoms [9]. The restrictions on life imposed by this chronic condition can be significant and, in terms of impact on quality of life, have been equated in more severe cases to those induced by dialysis and severe angina [10].

Tear film irregularity has been reported in DM, especially in association with that of extended disease duration and severity, as defined by stage of retinopathy [2, 11]. It has been postulated that damage to the microvasculature and denervation of the lacrimal gland may contribute to impaired lacrimation in DM [2, 7, 12]. Despite this neural link, few studies have explored whether the ocular surface alters in association with peripheral neuropathy [7, 13, 14], although anomalous innervation of the lacrimal gland has been reported in those with diabetic sensory neuropathy [2, 7].



(a) (b)

Figure 1: Representative examples of tear film—lipid layer grades: (a) grade 3: wave pattern; (b) grade 5: colour fringe pattern.

This prospective study sought to compare the tear film in DM with that of control subjects and examine the relationship of the preocular tear film with markers of peripheral neuropathy.

2. Materials and Methods

The study adhered to the tenets of the Declaration of Helsinki and was conducted with Regional Ethics Committee approval (NTX/09/12/122). Two hundred and seven patients, identified by a specialist endocrinologist (Author, Geoffrey D. Braatvedt) as having a history of type 1 DM, were invited to participate. A history of laser therapy for retinopathy, cumulative contact lens wear of ≥3 months, ocular surgery, ocular trauma, or ocular or systemic disease that may affect the ocular surface, or a diagnosis of peripheral neuropathy unrelated to DM precluded participation in the study. Consequently, 53 individuals with DM were deemed eligible and were willing to participate in the study. Forty healthy, nondiabetic volunteers (HbA_{1c} < 41 mmol/mol) were recruited as controls.

For each participant, a detailed ocular and general medical history, including smoking and alcohol use, was obtained. Dry eye symptoms were recorded and scored with the McMonnies dry eye questionnaire [15] and general ocular surface and eyelid examination was performed by slit-lamp biomicroscopy (Topcon Medical Systems, NJ, USA). Tear film interferometry with the Keeler Tearscope Plus (Keeler Ltd, Berkshire, UK) enabled tear film lipid patterns to be graded according to Guillon's classification [16]. Lipid patterns corresponding to increasing lipid layer thickness were subsequently assigned numerical lipid layer grades 0–5, for the purpose of statistical analysis, as 0 (absent), 1 (open meshwork), 2 (closed meshwork), 3 (wave), 4 (amorphous), or 5 (coloured fringes) (Figure 1). Tear film stability was recorded with the aid of the Tearscope Plus with the fine grid insert, as the noninvasive tear breakup time (NIBUT). An average of 3 readings was calculated for each measurement. The Phenol Red Thread (PRT) test (Zone-Quick, FCI Ophthalmics, Pembroke, MA, USA) provided an index of tear quantity. Central corneal sensitivity was measured using noncontact corneal aesthesiometry with the NCCA (Glasgow Caledonian University, UK) and adopting a double staircase method of threshold determination [17]. All ocular assessments were performed at a single visit, on the right eye only, with the exception of subjects reporting unilateral surgery or trauma to the right eye, in which case the left eye was examined. Tests were performed in the same order for each participant, from least to most invasive, to minimize the effect of reflex tearing.

Digital images of the central and peripheral retina were captured (Non-Mydriatic Retinal Camera DR-DGi, Canon Inc., USA) and graded according to the Early Treatment Diabetic Retinopathy Study (ETDRS) criteria [18] for diabetic retinopathy by an independent, fellowship-trained, medical retina specialist (Author, Monika Pradhan).

The DM group underwent neuropathic assessment by an experienced neurologist (Author, Dean Kilfoyle). An overall neuropathy score (total neuropathy score or TNS) was obtained from a previously validated and recognised combination of the symptomatic neuropathy score [19], a clinical neuropathic assessment by the neurologist, biothesiometry (quantitative sensory testing) on the medial malleolus and great toe, and nerve conduction study (NCS), with increasing score representing increasing severity of neuropathy [19].

Statistical analysis was undertaken with IBM SPSS v19.0 (Chicago, IL, USA). Regression analysis was performed between the results of the tear film assessment techniques, corneal sensitivity, diabetic retinopathy grading, and total neuropathy score. Positively skewed raw NIBUT data were logarithmically transformed prior to parametric statistical testing. One-factor ANOVA and Friedman tests were performed between variables with normally distributed and non-normally distributed data, respectively, to test differences between controls and DM groups. Pearson and Spearman correlation (2-tailed) analyses were performed for data that did and did not approximate normal distributions, respectively. A P value of less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Results. Patient characteristics including gender, age, ethnicity, and duration of diabetes are described in Table 1. All but two participants in the DM group self-identified as being of European descent while the control participants included

Table 1: Participant characteristics for the patients with type 1 diabetes and control group.

	Diabetes	Controls
Subjects (n)	53	40
M : F ratio	26 : 27	17 : 23
Age (years)	48.6 ± 11.8	44.3 ± 14.7
HbA_{1c} (mmol/mol)	61.3 ± 12.0	35.0 ± 2.5
Ethnicity		
European	51	19
Indian	0	9
Asian (excluding Indian)	1	8
Maori	1	0
Others	0	4
Mean diabetes Duration (years)	25.8 ± 11.4	
<10	5	
10–20	10	
21–31	22	
>31	16	

47.5% NZ European, 22.5% Indian, and 20% Asian (excluding Indian). The mean age of the DM group (49 ± 12 years) did not differ significantly from that of the control group (44 ± 15 years) ($P = 0.12$) and no significant differences in tear film characteristics were identified between the ethnic subgroups ($P > 0.05$).

Dry eye symptom scores and clinical findings were compared for the diabetes and control groups (Table 2). The mean dry eye symptom score was not statistically significantly different between the diabetes and control groups ($P = 0.33$). However, clinical tests showed significant differences between the two groups, with lipid layer grading ($P = 0.02$), NIBUT ($P < 0.0001$), and PRT test ($P = 0.01$) results all statistically, and clinically, significantly lower in the DM group. NIBUT was observed to be lower in females than in males in both the control ($P = 0.06$) and DM ($P < 0.01$) groups. The patients with DM exhibited reduced corneal sensitivity compared to controls ($P < 0.0001$) (corneal sensitivity and subbasal nerve density reported in detail elsewhere) [20]. The total neuropathy score (maximum possible score 40) ranged between 0 and 21 (5.3 ± 5.1) in the patients with diabetes.

Retinopathy was not observed in the majority of patients (60%) while 21% exhibited mild and 19% exhibited moderate DR. Regression analysis between interferometry, tear film stability, tear quantity, diabetic retinopathy grade, and total neuropathy score (Table 3) highlighted a positive relationship between lipid layer grading and both NIBUT ($r = 0.56$, $P < 0.01$) and tear quantity ($r = 0.38$, $P < 0.01$). Tear film stability was noted to be inversely related to total neuropathy score ($r = -0.29$, $P = 0.03$). The association between corneal sensitivity and total neuropathy score failed to reach statistical significance ($r = 0.24$, $P = 0.08$).

3.2. Discussion. Comparison of the status of the tear film between patients with type 1 DM and age-matched healthy control subjects demonstrated a significantly poorer tear film quality in diabetes. Lipid layer grade, tear film stability, and tear quantity (basal with minimal reflex tear secretion) [21] were all significantly reduced in patients with type 1 DM, confirming compromised protection of the ocular surface in patients with diabetes.

Dry eye symptoms were observed to increase in severity with advancing age in both groups, in the current study, consistent with reports in the literature [22, 23]. Interestingly, age-matched subjects in both groups reported similar dry eye symptom severity, despite clear differences in tear quality ($P < 0.0001$). This lack of difference in symptoms is believed to be related to the impaired sensitivity of the ocular surface in diabetes.

Tear film stability and lipid layer thickness have been shown to be influenced by gender [24], with older women tending to exhibit thinner and more contaminated lipid layers [25]. The current study also reported reduced tear film stability in females compared to males, both in the DM group ($P < 0.01$) and in the control group ($P = 0.06$). A decrease in circulating androgens in postmenopausal women is believed to play an important role in affecting meibomian gland function, supporting female gender as a risk factor for dry eye disease [26].

In addition to a reduction in tear film stability, several studies have reported a diminished tear secretion in the diabetic eye [7, 11, 27], as observed in the present research. This further degrades the quality of tear film in an already compromised diabetic eye.

Reduced tear film stability has been associated with the presence of superficial punctate keratitis [8]. Functional abnormalities of corneal innervation may contribute to the incidence of superficial punctate keratitis in these patients through its adverse effect on tear film instability [28]. An inverse association between corneal innervation and peripheral neuropathy has been reported previously [6, 29]. The current study supports this relationship, with a modest inverse correlation observed between NIBUT and total neuropathy score (a measure of peripheral neuropathy) ($r = -0.29$, $P = 0.03$).

Retinopathy grade was observed to be unrelated to tear film stability in the current study ($r = -0.03$, $P = 0.82$), contrary to previous observations [11, 30]. However, it should be noted that, as history of laser treated retinopathy was an exclusion criterion in the current study, patients with more than moderate retinopathy were generally ineligible to participate. This restriction in the range of disease severity in the cohort enrolled in the current study likely contributed to the absence of a relationship in our study.

In this New Zealand-based study, the control group comprised patients of a variety of ethnic backgrounds reflecting the multiethnic population [31]. Type 1 DM in New Zealand is predominantly reported in those with European (Caucasian) heritage [32] and this was observed in the present cohort. Although ethnicity has previously been shown to be a determinant of tear film stability [33], no significant

Table 2: Comparison of symptoms and tear characteristics for control and patient groups (mean/median), together with the significance of their differences (ANOVA/Friedmann). Noninvasive tear breakup time (NIBUT) values are extrapolated from logarithmically transformed data.

	Diabetes	Healthy controls	ANOVA/Friedmann (P values)
McMonnies questionnaire (mean ± SD)	8.8 ± 6.7	7.6 ± 4.6	0.33
Lipid layer thickness grade (median)	2	3	0.02
NIBUT (s) (mean ± SD)	6.0 ± 1.9	8.2 ± 2.5	<0.0001
Phenol red thread test (mm) (mean ± SD)	13.7 ± 4.7	16.3 ± 4.9	0.01
Corneal sensitivity threshold (mBAR)	1.3 ± 1.3	0.2 ± 1.3	<0.001

Table 3: Correlation analysis between age, diabetes duration, McMonnies questionnaire scores (DEQ), phenol red thread test (PRTT) (mm), tear film interferometry including lipid layer grade, stability (NIBUT), diabetic retinopathy grading, and total neuropathy score (TNS) in those with DM ($n = 53$).

	Correlation (r values)	Probability (P values)
Age versus NIBUT	−0.28*	0.05
Age versus TNS	0.41**	0.00
DEQ versus TNS	−0.05	0.73
Lipid layer thickness versus NIBUT	0.56**	0.00
Lipid layer thickness versus PRTT	0.39**	0.00
NIBUT versus diabetes duration	−0.29*	0.03
NIBUT versus TNS	−0.29*	0.03
NIBUT versus retinopathy grade	−0.03	0.82
PRTT versus TNS	0.01	0.96
PRTT versus retinopathy grade	−0.16	0.26

**Correlation is significant at the 0.01 level (2-tailed).
*Correlation is significant at the 0.05 level (2-tailed).

difference was observed between the ethnic subgroups of the control subjects in the present study.

There is potential for lipid, aqueous, and mucins, the major components of the tear film, to be adversely affected in patients with diabetes. The meibomian and lacrimal glands are responsible for the secretion of the lipid and aqueous portions of the preocular tear film, respectively. Meibomian glands are innervated by parasympathetic fibres with a smaller contribution from sympathetic and sensory neurons [34]. Disease or any damage to these neurons leads to dry eye in an animal model [35]. Two human studies have previously reported a compromised tear lipid layer in DM patients, as confirmed in the current study [8, 36]. Clinical observation of noncontiguous or absent lipid layers is associated with significantly increased tear film evaporation [37], one of the key factors in dry eye development [38].

Elevated expression of advanced glycation end products in the lacrimal gland has been postulated as a reason for changes in lacrimal gland function described in diabetes [12]. A reduction in goblet cell numbers, compromising mucin quantity, may also contribute to the tear film instability observed in diabetes [7, 39]. Goblet cell loss in those with diabetic peripheral neuropathy and poor metabolic control has been previously reported [7].

The reduced tear production identified in patients with diabetes ($P = 0.01$) in the current study lends support to the concept that lacrimal gland function might be adversely affected by a neuropathic mechanism [40], resulting in dysfunction of the ocular surface secretory glands via their innervation. Such dysfunction could arise from a peripheral neuropathy involving the afferent sensory nerves from the ocular surface affecting corneal sensitivity and the autonomic (efferent) nerves responsible for innervating the tear-component secreting glands and the lacrimal and meibomian glands [35, 40, 41]. This is supported by the current results of reduced corneal sensitivity; however the previously reported association between corneal sensitivity and peripheral neuropathy failed to reach statistical significance in the current study ($r = 0.24$, $P = 0.08$) [42]. Reduced tear secretion has previously been identified in patients with type 2 diabetes relative to healthy controls [8, 11].

4. Conclusion

In summary, the current study confirms the underlying threat to ocular surface health in patients with type 1 DM compared to control subjects. The reduction in tear production in patients with DM and the association between reduced tear film stability and diabetic peripheral polyneuropathy add credence to the hypothesis that diabetic peripheral neuropathy is associated with, or directly affects, secretory lacrimal gland function [43]. Hence, patients with DM, particularly the older

female, are at a greater risk of dry eye and compromised preocular tear film.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Early Detection of Atrophy of Foot Muscles in Chinese Patients of Type 2 Diabetes Mellitus by High-Frequency Ultrasonography

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The aim of this study was to evaluate the diagnostic value of high-frequency ultrasonography in detecting atrophy of foot muscles in Chinese patients of type 2 diabetes mellitus (T2DM). Chinese patients of T2DM with ($n = 56$) or without ($n = 50$) diabetic peripheral neuropathy (DPN) and the control subjects ($n = 50$) were enrolled. The nondominant foot of all subjects was examined with high-frequency ultrasonography. The transverse diameter, thickness, and cross-sectional area of the extensor digitorum brevis muscle (EDB) and the thickness of the muscles of the first interstitium (MILs) were measured. The results showed that the ultrasonographic transverse diameter, thickness, and cross-sectional area of EDB and the thickness of MILs in patients of T2DM with DPN were significantly smaller than those in patients of T2DM without DPN (all $P < 0.01$) and those in the control subjects (all $P < 0.01$). The transverse diameter and cross-sectional area of the EDB and thickness of MILs in patients of T2DM without DPN were significantly smaller than those of the control subjects (all $P < 0.01$). In conclusion, the atrophy of foot muscle in Chinese T2DM patients can be detected by high-frequency ultrasonography. Notably, ultrasonography may detect early atrophy of foot muscles in patients without DPN.

1. Introduction

Diabetes mellitus is a major health problem which affects approximately 170 million people worldwide in 2001 and estimated more in the future [1, 2]. Importantly, developing countries with rapid change of life style, such as China, are facing the problem as well [3]. The population of type 2 diabetes mellitus (T2DM) especially increases dramatically in the Chinese population [4–6]. Diabetic neuropathy is a common and chronic complication of diabetes, which is responsible for most limb amputations and morbidity partly because of foot muscles atrophy [7, 8]. Notably, the extent of foot muscles atrophy is also a reliable measurement of the neuropathic process [9]. Thus, it is important to determine the degree of foot muscles atrophy to help evaluate the neuropathic process and decide the corresponding strategies of timely therapy [10, 11].

Foot muscles atrophy in the Danish diabetic patients of both type 1 and type 2 with diabetic peripheral neuropathy (DPN) has been detected with ultrasonography [12, 13]. As far as we know, the method has not been tested in Chinese diabetic patients of T2DM by now. Additionally, the sensitivity of the detection needs to be tested in the Chinese since the muscle size of them is smaller than that of the Caucasians such as the Danes [14]. The present study explored whether ultrasonography can detect the foot muscles atrophy in Chinese diabetic patients of T2DM including those before and after the concurrency of DPN.

2. Materials and Methods

2.1. Participants. One hundred and six Chinese inpatients of T2DM (56 males, 50 females, mean age of 61 ± 6 years, range of 38–78 years) were recruited from the Department of Endocrinology of the Second Affiliated Hospital of Dalian Medical University. All patients were diagnosed by experienced physicians in accordance with the World Health Organization guidelines of diagnosis and classification [15]. Patients were then divided into two groups according to the diagnosis of DPN. The criteria of DPN are referring to the principles of the San Antonio Consensus criteria [16]. Neuropathy symptom score and neuropathy disability score were evaluated [17, 18]. In the group of patients with DPN, there were fifty-six patients (30 males, 26 females, mean age of 63 ± 7 years, range of 44–77 years); in the group of patients without DPN, there were fifty patients (26 males, 24 females, mean age of 59 ± 10 years, range of 38–78 years). Fifty matched control subjects (25 males, 25 females, mean age of 59 ± 7 years, range of 49–78 years) were enrolled among the hospital staff. All subjects gave informed consent to the study which was approved by the local ethics committee.

All subjects were able to walk by themselves without a history of foot surgery or arterial insufficiency of the lower extremities. Subjects with open lesions on their feet, peripheral vascular disease (symptoms of claudication and absence of peripheral pulses, ankle brachial index [ABI] < 0.7) [19], severe cardiac or lung disease, cancer, acute or chronic musculoskeletal disease, other neurological diseases, other endocrine disorders, or symptomatic peripheral artery disease and those who were carrying out motor function rehabilitation were excluded. Sports professionals were excluded.

2.2. Data Collection. Both vibration perception threshold (VPT) by biothesiometry and pressure perception using Semmes-Weinstein monofilaments (SWM) have been used to measure the extent of neuropathy [20]. Age, gender, BMI, diabetic duration, HbA1C, and ankle brachial index were recorded and obtained.

2.3. Ultrasonographic Examination. All ultrasonographic examinations were performed by the same examiner using a scanner with a linear array real-time high-frequency ultrasonic probe (GE LOGIQ E9, 9–15 MHz). The subjects were placed in a supine position with the nondominant foot placed on a plastic ramp to keep the ankle joint in a neutral position, in case of the adverse effect of possibly excessive extension or contraction. The examiner detected two groups of muscles, including the extensor digitorum brevis muscle (EDB) and the muscles between the first and second metatarsal bone (MILs). The transverse diameter, thickness, and cross-sectional area of the EDB (with the 15 MHz beam) and thickness of the MILs (with the 9 MHz beam) were detected. The examination methods of either muscle group were implemented as described before [13]. The Patients with obvious foot muscles atrophy were asked to perform voluntary contraction of muscles to help define the muscle before examination. The ultrasonograms were downloaded to computers and each parameter was measured five times by the included measurement and analysis package (GE LOGIQ E9). After exclusion of the extreme values, the average of the remaining values was used for further analysis.

2.4. Statistical Analysis. All data was analyzed with professional software of statistics (SPSS version 13.0, SPSS Inc., Chicago, USA). The quantitative data was represented as mean and standard deviation ($\overline{x} \pm$ SD). The variable coefficients (CV) of values of each parameter in control subjects were calculated with the formula CV = SD/$\overline{x}$ × 100%. Multiple mean comparison was analyzed by the one-way analysis of variance (ANOVA) followed by post hoc LSD test. $P < 0.05$ was considered statistically significant.

Table 1: Characteristics of the study groups.

	Control group	T2DM without DPN group	T2DM with DPN group
Number of cases	50	50	56
Age (years)	59 ± 7	59 ± 10	63 ± 7
Sex (male/female)	25/25	26/24	30/26
BMI (kg/m^2)	24.7 ± 5.8	27.3 ± 7.1	28.1 ± 4.7
Diabetes duration (years)*	—	6 ± 3	18 ± 5
HbA1C (%)*	—	7.6 ± 1.9	8.8 ± 2.3
Ankle brachial index†	1.1 ± 0.1	1.1 ± 0.2	0.9 ± 0.2
NSS†	0 ± 0	2 ± 2	4 ± 2
NDS‡	0 ± 0	2 ± 1	12 ± 7
VPT‡	8 ± 2	14 ± 8	35 ± 17
SWM‡	3.79 ± 0.52	4.12 ± 0.63	6.55 ± 0.78

Data are means ± SD. *$P < 0.01$ for T2DM without DPN versus T2DM with DPN; †$P < 0.01$ for control and T2DM without DPN versus T2DM with DPN; ‡$P < 0.001$ for control and T2DM without DPN versus T2DM with DPN. NSS: neuropathy symptom score; NDS: neuropathy disability score; VPT: vibration perception threshold; SWM: Semmes-Weinstein monofilaments.

3. Results

The baseline demographic data of study population is shown in Table 1. Average age, gender, and BMI were similar among three groups. There were significant differences in diabetic duration and HbA1C between the T2DM group without DNP and the T2DM group with DNP (all $P < 0.01$). There were significant differences in ankle brachial index, NSS (neuropathy symptom score), NDS (neuropathy disability score), VPT (vibration perception threshold), and SWM (Semmes-Weinstein monofilaments) between the control group, the T2DM group without DNP, and the T2DM group with DNP ($P < 0.01$ for ankle brachial index and NSS; $P < 0.001$ for NDS, VPT, and SWM).

Table 2: Measured foot muscle atrophy in Chinese diabetic patients and the matched control subjects.

	Control group ($n = 50$)	T2DM without DPN group ($n = 50$)	T2DM with DPN group ($n = 56$)
EDB transverse diameter (mm)	75.85 ± 9.03	66.93 ± 9.28$^{\&}$	53.95 ± 11.05$^{*\#}$
EDB thickness (mm)	7.16 ± 0.94	6.91 ± 0.97	5.61 ± 0.90$^{*\#}$
EDB cross-sectional area (mm^2)	165.42 ± 32.86	138.10 ± 39.26$^{\&}$	90.40 ± 29.90$^{*\#}$
MILs thickness (mm)	34.32 ± 1.93	32.16 ± 2.86$^{\&}$	30.07 ± 2.85$^{*\#}$

$*$ and & stand for comparison with the control group, $P < 0.01$; # stands for comparison with the group of T2DM without DPN, $P < 0.01$.

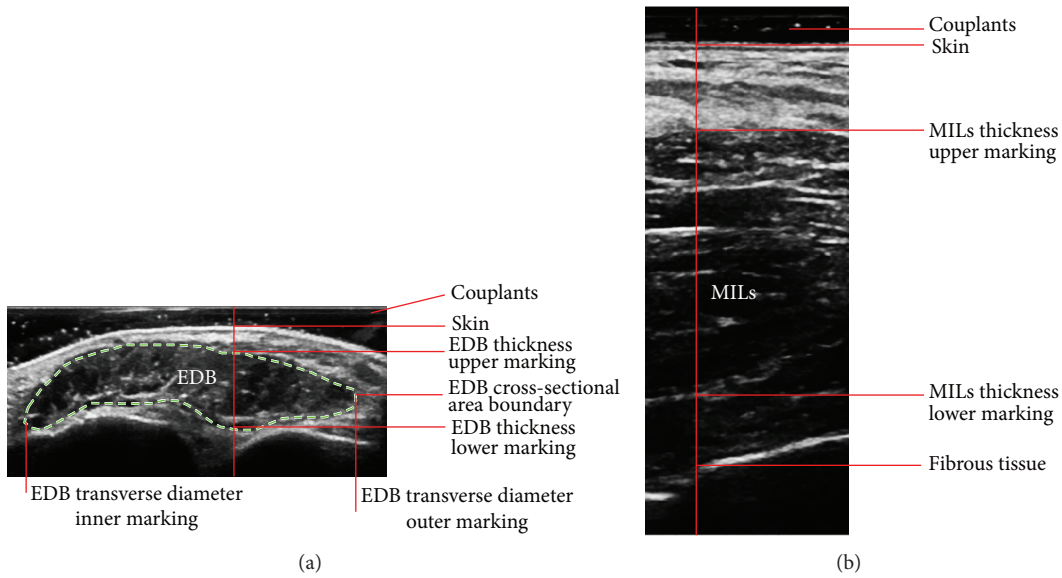

Figure 1: Ultrasonographic images of the extensor digitorum brevis muscle (EDB) and the muscles of the first interstitium (MILs). (a) A representative ultrasonic image of EDB, along with the enthesis (indicating lines with annotations) and boundary (dashed line) of the measured transverse diameter, thickness, and cross-sectional area. (b) A representative ultrasonic image of MILs and the enthesis (indicating lines with annotations) of the measured thickness.

In the ultrasonography, the fascicles were shown as hypoechogenicity or medium echogenicity, surrounded by epimysium, fascia, and adipose tissue shown as hyperechogenicity of strips and streaks (Figure 1). The measured boundary of muscles was defined with the inner edge of fascia (see labels in Figure 1). The ultrasonographic transverse diameter, thickness, and cross-sectional area of the EDB and the thickness of MILs in patients of T2DM with DPN were significantly smaller than those in patients of T2DM without DPN (all $P < 0.01$) and those in the control subjects (all $P < 0.01$). The transverse diameter and cross-sectional area of the EDB and thickness of MILs in patients of T2DM without DPN were significantly smaller than those of the control subjects (all $P < 0.01$). Data of ultrasonographic measurement are shown in Table 2. Among the changes of parameter values in patients with DPN, the cross-sectional area of EDB decreased the most, for example, to 65.46 ± 21.65% of that in patients without DPN, while the transverse diameter of EDB decreased to 80.61 ± 16.59%, the thickness of EDB to 81.19 ± 13.02%, and the thickness of MILs to 93.50 ± 8.86% (graph in Figure 2).

The CVs were 11.9% for the transverse diameter of the EDB, 13.13% for the thickness of the EDB, 19.86% for the cross-sectional area of the EDB, and 5.62% for the thickness of the MILs.

4. Discussion

The present study is the first exploration of ultrasonographic detection of foot muscle atrophy in Chinese diabetic patients of T2DM with or without DPN as far as we know. The results have shown that foot muscle atrophy occurs in diabetic patients including those without DPN, which can be detected by ultrasonographic examination and measurement on EDB and MILs. The cross-sectional area of EDB may be the most sensitive parameter.

Diabetic patients have pathological changes of metabolism and microvessels along with later neuropathy, which may cause muscle atrophy. It involves complicated cellular and molecular mechanisms, including apoptosis and abnormality of ubiquitin-proteasome and lysosome systems [21, 22]. DPN especially may induce movement disorders in distal extremities such as decrease of muscle strength of foot, resulting in nonhealing ulceration and tragic amputation [23]. Thus, the foot muscle atrophy in

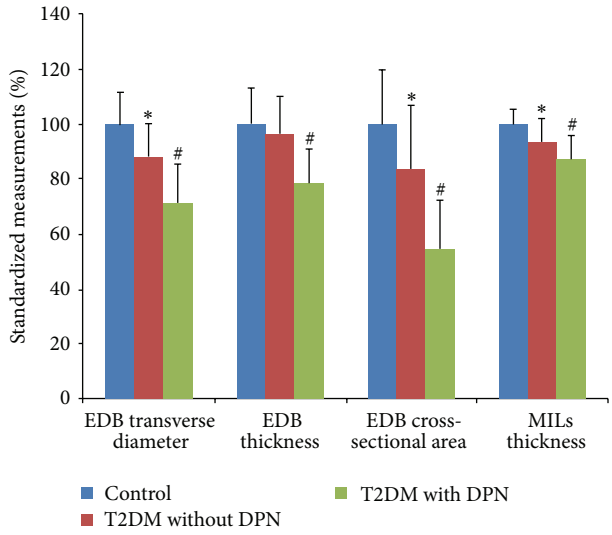

Figure 2: Standardized measurements of the extensor digitorum brevis muscle (EDB) and the muscles of the first interstitium (MILs). The graph shows the standardized measurements when the measured values of the control group are normalized as 100. ∗ indicates $P < 0.01$ when compared with the control group. # stands for $P < 0.01$ when compared with the T2DM without DPN group.

diabetic patients is progressive, possibly early before the concurrency of DPN, which may be worsened by DPN and can cause serious consequences eventually. It is of important clinical significance to have early diagnosis of the atrophy in the patients. Practically, physical examination can find the evident muscular dystrophy and foot deformity in the patients at the late stages, but unlikely the early subtle changes [23]. Neurophysiological detection targets the electrophysiological conduction deficiency of DPN, rather than muscle atrophy. Magnetic resonance technique has excellent performance to detect the early muscle atrophy, but it is time-consuming, expensive, and inconvenient for bedside examination [24], while ultrasonography has the superiority over the above flaws.

The present study demonstrated that high-frequency ultrasonography is able to acquire clear images of foot muscle of patients with T2DM for the subsequent measurement of multiple parameters. The analysis of data shows that most measured values are smaller in the diabetic patients than those in the control subjects, and the values are even smaller in the patients with DPN than those in the patient without DPN. It suggests that high-frequency ultrasonography can detect the foot muscle atrophy not only at the late stage with DPN but also at the early stage without DPN. It also supports the early occurrence of foot muscle atrophy in diabetic patients [25].

Among the measured parameters, the cross-sectional area of EDB decreased the most. It is consistent with the fact that EDB is the earliest to shrink [26] and also implicates that EDB may serve as the best optional muscle group for the early detection of foot muscle atrophy, which is critical in the screening among large population. The early detection may provide an opportunity for the timely prevention of the foot muscle atrophy, such as optimal footwear [27, 28].

Technically, ultrasonography has been a matured application for the detection of muscle atrophy [29]. The important thing is to ensure the reproducibility of tissue measurement [30, 31]. Our study kept strict accordance with the previously described methods [13] and tested the repeatability of the measurement by analyzing CV of the parameters. Remarkably, our results have comparable reproducibility (all CVs below 4%) to that in the former study (all CVs below 6%) [13]. Larger population included in the detection of our study might also contribute to the good reproducibility.

The sensitivity of the detection may be affected by several factors. For example, the ethnic differences [32] and experiences of sports or rehabilitation training [33] may have affected the baseline of measured values in the subjects, which is critical for the following judgment of the changes. Our results suggested that the measured size of foot muscles in Chinese population may be smaller than that in Caucasians including the Danes [13]. To alleviate the risk of nondetectability, a larger population was included in our study than before [13]. This strategy successfully helped the discovery of the muscle atrophy not only in diabetic patients but also in patients without DPN. To eliminate the influence of training, individuals with such experience have been excluded after the inquiry.

Our study has limitations though. For instance, we have not coordinated the extent of possible atrophy with the specific disease course to identify the detailed relationship [12], which may help more in the early detection [23, 29]. The previous studies [12, 13] and ours used the two-dimensional images of ultrasonography for analysis. Although there is a good correlation among the thickness, cross-sectional area, and volume of muscles [34], the 3D imaging combined with the unbiased stereological analysis is expected to provide the information of volume change more directly [35].

5. Conclusion

Our data show that the atrophy of foot muscle in Chinese T2DM patients, especially at the early stage without DPN, can be detected by high-frequency ultrasonography. The cross-sectional area of EDB is suggested to be the most sensitive parameter, particularly for a fast screening in a large population. This convenient and sensitive detection may help timely prevention of diabetic foot and improve the prognosis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Xiaohui Wang and Liang Chen contributed equally to this work.

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Clinical Study

Study of Postprandial Lipaemia in Type 2 Diabetes Mellitus: Exenatide versus Liraglutide

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Therapeutic approaches based on the actions of the incretin hormone GLP-1 have been widely established in the management of T2DM. Nevertheless, much less research has been aimed at elucidating the role of GLP-1 in lipid metabolism and in particular postprandial dyslipidemia. Exenatide and liraglutide are two GLP-1 receptor agonists which are currently available as subcutaneously administered treatment for T2DM but their chronic effects on postprandial lipaemia have not been well investigated. The aim of this study is to examine the effect of treatment with either liraglutide or exenatide for two weeks on postprandial lipaemia in obese subjects with T2DM. This study was a single-center, two-armed, randomized, controlled 2-week prospective intervention trial in 20 subjects with T2DM. Patients were randomized to receive either liraglutide or exenatide treatment and underwent a standardized meal tolerance test early in the morning after 10 h fast at baseline (visit 1, beginning of treatment) and after a two-week treatment period (visit 2). Exenatide and liraglutide both appear to be equally effective in lowering postprandial lipaemia after the first administration and after a two-week treatment. The mechanisms which lead to this phenomenon, which seem to be independent of gastric emptying, are yet to be studied.

1. Introduction

The growing incidence of type 2 diabetes mellitus (T2DM) is a major problem in the modern world [1]. Most individuals with T2DM have insulin resistance and are at increased risk of developing cardiovascular disease (CVD) [2]. Diabetic dyslipidemia contributes to the excess morbidity and mortality in T2DM [2] and postprandial triglyceridemia is a distinct component of diabetic dyslipidemia [3]. Postprandial triglyceridemia is an independent risk factor for CVD in individuals with and without T2DM [4–6].

Obesity is associated with insulin resistance, which in turn is linked to atherogenic dyslipidemia, which postprandial hyperlipidemia is a major component of [7]. Atherosclerotic cardiovascular disease is one important consequence of the obesity pandemic that currently affects more than a billion people worldwide [8].

Thus, therapeutic approaches aiming to reduce postprandial lipid concentrations may reduce the cardiovascular mortality in patients with T2DM [9, 10].

Glucagon-like peptide-1 (GLP-1) is a gut incretin hormone secreted in response to nutrient ingestion [11]. It has several physiological effects mediated by the widely expressed GLP-1 receptor. Following binding to and activation of the GLP-1 receptor in pancreatic β cells, insulin secretion is elicited in a glucose dependent manner.

GLP-1 also delays gastric emptying and induces satiety, thus decreasing energy intake, which in turn leads to weight loss [12].

Therapeutic approaches based on the actions of the incretin hormone glucagon-like peptide GLP-1 have been widely established in the management of type 2 diabetes [13–15].

Interestingly, much less research has been aimed at elucidating the role of GLP-1 in lipid metabolism and in particular postprandial dyslipidemia, although preclinical models have provided some clues in this regard. Acute intravenous administration of the incretin hormone GLP-1 has been shown to lower postprandial triglyceride levels in healthy volunteers [16]. There are limited human data investigating the chronic effects of GLP-1 receptor agonists on postprandial triglyceride and lipoprotein concentrations.

Currently, two GLP-1 receptor agonists are available as subcutaneously administered treatment for T2DM, exenatide and liraglutide.

Long-term clinical trials with liraglutide or exenatide versus placebo have shown their ability to improve glycaemic control and reduce body weight [17], but their chronic effects on postprandial lipaemia have not been well investigated.

2. Materials and Methods

This was a single-center, randomized, interventional study in 20 patients with T2DM. All study subjects gave their informed consent. The study was conducted in the Diabetic Centre of the "Konstantopouleio" General Hospital, Nea Ionia, Athens, Greece, between April 2013 and November 2013. The study included obese men and women aged 18–80 years with T2DM who were treated with diet and exercise with or without a stable dose of metformin for at least 30 days prior to the start of the protocol. Patients were deemed obese if their body mass index (BMI) equaled or exceeded 30 kg/m^2.

Main exclusion criteria included impaired renal function (creatinine clearance < 1.0 mL/s), known clinically significant active CVD, alcoholism/drug abuse, treatment with corticosteroids within 2 months, treatment with an investigational drug within 30 days, or current treatment with drugs known to affect gastrointestinal motility. None of the participants had any abnormalities, in terms of either (1) anaemia (haemoglobin < 120 g/L) or (2) an elevation of liver enzymes (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and γ-glutamyltransferase) to levels higher than double the respective normal value. Women who were pregnant or intended to become pregnant during the study and women who were breastfeeding were also excluded. Patients were excluded if they had clinically important medical conditions or had used thiazolidinediones, sulfonylureas, meglitinides, a-glucosidase inhibitors, pramlintide, exogenous insulin, lipid-lowering drugs, or weight-loss drugs within the prior 2 months. None of the participants had ever used DPP-4 inhibitors or GLP-1 analogues, nor did they have a history of gastrointestinal disorders or had previously undergone abdominal surgery. All the participants were nonsmokers.

Patients underwent a standardized meal tolerance test at baseline (visit 1—beginning of treatment) and after a two-week treatment period (visit 2). Meals provided for each patient were identical at the baseline and week-2 assessments. The standardized meal LIPOTEST (D.GENOMERES Medical Research, Athens, Greece) [18] was given in the form of 115 g powder diluted in water (150 mL water, final volume as mousse 265 mL). The meal consists of 75 g of fat, 25 g of carbohydrates, and 10 g of protein.

After at least a 10-h overnight fast, exenatide or liraglutide was administered 30 minutes prior to the standard meal, as described in previous studies. All participants were advised to maintain their usual dietary habits and to avoid strenuous exercise before the experiments. Each patient received a daily injection with either liraglutide or bidaily injection of exenatide according to the group each patient was randomized to. Individuals randomized to liraglutide underwent a weekly dose escalation, starting at 0.6 mg/day of subcutaneous (sc) injection for the first week followed by 1.2 mg/day for the second week. Exenatide treatment consisted of a 5 mg sc injection twice daily (bid) for the first week followed by 10 mg sc bid for the second week.

The patients were monitored at baseline as well as two weeks after the beginning of treatment. At each of the two visits the following parameters were recorded for each patient: serum cholesterol, high density lipoproteins (HDL), low density lipoproteins (LDL), triglycerides, insulin, and glucose. Each parameter was measured at 0 (fasting), 120, and 240 minutes after the ingestion of the lipid meal.

Furthermore the patient's body mass index (BMI), waist circumference, thigh circumference, and HbA1c were recorded.

For the statistical analysis we used the Mann-Whitney independent samples U test and Fisher's exact test to test for differences in means of the baseline characteristics between patients in the exenatide and liraglutide groups. ANOVA and ANCOVA for repeated measurements were performed to test the timing effect of the studied parameters after the test meal and the differences between exenatide and liraglutide at visit 1 and at visit 2, respectively. The Greenhouse-Geisser adjustment was used when the sphericity assumptions were not fulfilled. Postprandial responses over the 240-minute period were calculated as the area under the curve (AUC) using the trapezoid rule.

3. Results

The baseline characteristics of the patients in the two study groups are illustrated in Tables 1 and 2.

There was no statistically significant difference in age, HbA1c, waist-to-hip ratio, and BMI between the exenatide and liraglutide groups.

The fasting values for different parameters of the patients in the exenatide group and the liraglutide group for visits 1 and 2 are shown in Tables 3 and 4.

There was an increase in the postprandial total cholesterol levels in patients treated with both exenatide and liraglutide at visit 1, which was statistically significant. Postprandial triglycerides also showed an increase, but that did not reach statistical significance. HDL levels remained stable in the two measurements after the standard meal. Glucose in both

Table 1: Demographic and clinical characteristics of the study subjects stratified according to treatment.

Characteristics	Exenatide ($n = 10$)	Liraglutide ($n = 10$)	P
Age (years)	45.2 ± 11.4	54.6 ± 12.9	0.143
Diabetes duration (years)	2.40 ± 2.17	2.50 ± 2.32	0.922
Hypertension	3 (yes)/7 (no)	4 (yes)/6 (no)	1.000*
Metformin use	9 (yes)/1 (no)	10 (yes)/0 (no)	1.000*
HbA1c (mmol/mol)	41.53 ± 10.17	42.74 ± 6.97	0.912
WHR (waist-to-hip ratio)	1.01 ± 0.13	0.98 ± 0.08	0.393
BMI (kg/m^2)	37.18 ± 5.82	37.11 ± 5.09	1.000
HbA1c (mmol/mol)	41.53 ± 10.17	42.73 ± 6.97	0.915

Data are means ± SD. (P indicates the result of the Mann-Whitney test between the two groups; *the result of Fisher's exact test for differences between the two groups).

Table 2: Baseline measurements of the study subjects stratified according to treatment.

Characteristics	Exenatide ($n = 10$)	Liraglutide ($n = 10$)	P
Glucose (mg/dL)	7.34 ± 2.73	6.69 ± 0.94	0.971
Total cholesterol (mg/dL)	5.05 ± 1.42	5.15 ± 1.19	0.853
Triglycerides (mg/dL)	3.03 ± 4.69	2.42 ± 2.97	0.853
HDL (mg/dL)	1.03 ± 0.33	1.19 ± 0.45	0.481
LDL (mg/dL)	3.00 ± 1.26	2.87 ± 0.71	1.000
Insulin (mU/L)	159.86 ± 84.58	152.07 ± 49.74	0.739

Data are means ± SD. (P indicates the result of the Mann-Whitney test between the two groups).

Table 3: Fasting and postprandial profiles of the measured parameters in patients receiving exenatide and liraglutide on visit 1.

Parameters	0	120	240	P	P^*
Triglycerides (mmol/L)					
Exenatide	3.03 ± 4.69	3.18 ± 4.24	3.48 ± 4.23	0.185	0.147
Liraglutide	2.42 ± 2.97	2.47 ± 2.47	2.53 ± 2.59	0.628	
Total cholesterol (mmol/L)					
Exenatide	5.05 ± 1.42	5.06 ± 1.41	5.20 ± 1.50	0.048	0.232
Liraglutide	5.15 ± 1.19	5.00 ± 1.00	5.24 ± 1.16	0.016	
HDL (mmol/L)					
Exenatide	1.03 ± 0.33	1.04 ± 0.30	1.03 ± 0.33	0.622	0.392
Liraglutide	1.19 ± 0.45	1.17 ± 0.44	1.19 ± 0.45	0.597	
LDL (mmol/L)					
Exenatide	3.00 ± 1.26	3.00 ± 1.23	3.04 ± 1.29	0.041	0.461
Liraglutide	2.87 ± 0.71	2.80 ± 0.63	2.85 ± 0.63	0.187	
Glucose (mmol/L)					
Exenatide	7.33 ± 2.73	6.50 ± 1.44	5.91 ± 1.84	0.032	0.803
Liraglutide	6.69 ± 0.93	6.06 ± 1.20	5.08 ± 0.53	<0.0001	
Insulin (pmol/L)					
Exenatide	159.86 ± 84.58	232.67 ± 131.82	153.00 ± 117.10	0.006	0.424
Liraglutide	152.07 ± 49.74	208.39 ± 111.16	105.07 ± 63.80	0.002	

Data are means ± SD. P indicates the result of ANOVA for repeated measurements within each group (P value for the effect of time); P^* indicates the result of ANOVA for repeated measurements between the two groups (exenatide versus liraglutide) (time × group interaction).

groups and LDL in the liraglutide-treated patients showed a decrease in their levels, the former being statistically significant, the latter not. In contrast LDL in the exenatide group showed a marginal increase that was statistically significant. Postprandial insulin reached a peak value at 120 minutes after the mean to return to lower level at 240 minutes in both groups. There was no difference between any of the parameters, when comparing the exenatide and liraglutide groups.

At visit 2 triglycerides, HDL, and LDL increased at the two postprandial measurements as did the total cholesterol in the liraglutide group. To the contrary, total cholesterol in

Table 4: Fasting and postprandial profiles of the measured parameters in patients receiving exenatide and liraglutide on visit 2.

Parameters	0	120	240	P	P^*
Triglycerides (mmol/L)					
Exenatide	2.16 ± 2.42	2.42 ± 2.37	2.50 ± 2.13	0.040	0.523
Liraglutide	1.72 ± 1.00	1.92 ± 0.94	1.89 ± 0.88	0.030	
Total cholesterol (mmol/L)					
Exenatide	4.73 ± 1.22	4.72 ± 1.22	4.38 ± 1.87	0.594	0.464
Liraglutide	4.69 ± 1.13	4.70 ± 0.01	4.82 ± 1.03	0.161	
HDL (mmol/L)					
Exenatide	1.00 ± 0.28	1.01 ± 0.28	1.05 ± 0.28	0.017	0.493
Liraglutide	1.19 ± 0.41	1.17 ± 0.42	1.20 ± 0.43	0.572	
LDL (mmol/L)					
Exenatide	2.91 ± 1.13	2.92 ± 1.17	3.10 ± 1.29	0.002	0.105
Liraglutide	2.75 ± 0.97	2.71 ± 0.92	2.81 ± 0.90	0.150	
Glucose (mmol/L)					
Exenatide	6.42 ± 1.46	6.80 ± 2.36	6.07 ± 1.88	0.069	0.710
Liraglutide	6.02 ± 1.15	6.15 ± 1.50	5.30 ± 0.61	0.066	
Insulin (pmol/L)					
Exenatide	150.75 ± 87.46	233.15 ± 182.40	172.93 ± 175.63	0.143	0.081
Liraglutide	144.53 ± 72.94	413.56 ± 350.00	127.91 ± 84.20	0.026	

Data are means ± SD. P indicates the result of ANOVA for repeated measurements within each group (P value for the effect of time); P^* indicates the result of ANOVA for repeated measurements between the two groups (exenatide versus liraglutide) (time × group interaction).

Table 5: Total AUC values in patients receiving exenatide and liraglutide on visits 1 and 2.

Parameters	Exenatide	Liraglutide	P
First visit			
Triglycerides (mmol/L·min)	771.50 ± 1041.54	593.39 ± 628.76	0.290
Total cholesterol (mmol/L·min)	1221.13 ± 343.35	1223.00 ± 258.93	0.140
HDL (mmol/L·min)	248.80 ± 74.93	284.07 ± 106.98	0.306
LDL (mmol/L·min)	716.39 ± 300.32	679.56 ± 155.12	0.472
Glucose (mmol/L·min)	1575.42 ± 408.90	1433.90 ± 206.56	0.364
Insulin (pmol/L·min)	46692.36 ± 26383.91	40435.20 ± 19466.77	0.151
Second visit			
Triglycerides (mmol/L·min)	569.18 ± 556.38	446.46 ± 224.27	0.307
Total cholesterol (mmol/L·min)	1112.75 ± 266.78	1134.73 ± 249.96	0.850
HDL (mmol/L·min)	244.76 ± 67.94	283.61 ± 100.85	0.305
LDL (mmol/L·min)	711.89 ± 280.85	660.29 ± 221.26	0.089
Glucose (mmol/L·min)	1565.43 ± 477.52	1416.92 ± 252.87	0.762
Insulin (pmol/L·min)	47399.76 ± 35383.95	65973.24 ± 45256.94	0.174

Data are means ± SD. P indicates the result of the Mann-Whitney U test.

the exenatide group showed a decrease that was not statistically significant. Glucose levels showed a similar decrease in the first visit and insulin levels peaked at 120 minutes to level off at 240 minutes after the lipid meal. There was no difference for any of the parameters between the exenatide and liraglutide groups.

The AUC values for each parameter of the patients in the exenatide and liraglutide groups at visits 1 and 2 are shown in Table 5. There was no statistically significant difference for any of those between exenatide and liraglutide. The results remained the same even after correcting for possible confounding factors (age, WHR, HbA1c, and BMI).

4. Discussion

Acute intravenous administration of the incretin hormone GLP-1 has been shown to lower postprandial triglyceride levels in healthy volunteers [16]. The precise mechanism is yet to be determined, as many factors can influence circulating triglyceride concentrations [5, 19].

Short-term treatment with vildagliptin and sitagliptin, selective DPP-4 inhibitors, has been shown to reduce postprandial lipaemia in T2DM [20, 21], presumably by inhibiting the inactivation of endogenous GLP-1 by DPP-4.

Also, treatment with the GLP-1 receptor agonist exenatide reduces postprandial triglyceride and lipoprotein concentrations in T2DM, in the short term [22]. Previous studies proved that even a single dose of exenatide significantly lowers postprandial triglycerides levels [23]. The effect of exenatide on postprandial lipaemia may be influenced by several mechanisms mediated by GLP-1 receptor signaling, as well as by delayed gastric emptying [24].

Recently, Hermansen et al. investigated the effects of steady-state liraglutide 1.8 mg versus placebo on postprandial plasma lipid concentrations after 3 weeks of treatment in patients with T2DM. The conclusion of this study was that liraglutide treatment in patients with T2DM significantly reduced postprandial excursions of triglyceride and apolipoprotein B48 after a fat-rich meal. Reductions in postprandial glucose and glucagon responses, as well as in LDL and total cholesterol concentrations from baseline, were also observed with liraglutide. The effects of liraglutide on postprandial lipaemia appeared to be independent of gastric emptying [25].

The present study is the first to compare the effects of exenatide and liraglutide on postprandial lipaemia both in short and in long term in obese patients with T2DM.

Although exenatide and liraglutide share the same basic mechanisms of action, differences in pharmacokinetic and pharmacodynamic characteristics translate into differential effects on parameters of fasting and postprandial glycaemia [26] and presumably of postprandial lipaemia.

A deceleration of gastric emptying by GLP-1 has been demonstrated in patients with type 2 diabetes and healthy individuals [27–29]. It has also been reported that the GLP-1 induced deceleration of gastric emptying is significantly diminished already after 5 h of continuous infusion compared with its initial effects. It has also been shown that postprandial glucose control by GLP-1 is attenuated by its chronic administration [30].

GLP-1 seems to inhibit gastric emptying primarily through inhibition of the vagal nerve [31], but this mechanism is subject to rapid tachyphylaxis after chronic GLP-1 exposure [30].

The difference in the duration of action between bidaily exenatide and liraglutide influences gastric emptying. Therefore, delayed gastric emptying might be attenuated with long-acting GLP-1 analogs such as liraglutide compared to short-acting GLP-1 analogs such as exenatide [32]. Indeed, gastric emptying time was more prolonged with exenatide compared to long-acting release exenatide (LAR), which is administered once weekly, in patients with type 2 diabetes [33]. In addition to the different durations of action, different immunogenicity profiles across different classes of GLP-1 analogs could affect drug efficacy and safety profiles [34].

In a recent study, the acute and chronic effects of exenatide twice daily and liraglutide on gastric emptying were examined in rats. Gastric emptying was assessed using a standard acetaminophen release assay. After the acute test, rats were administered either exenatide bidaily or daily liraglutide for 14 days. On day 14, the gastric emptying rate was reassessed. While both compounds exerted robust acute reductions in gastric emptying, the effect was markedly diminished following 14 days of liraglutide administration. In contrast, exenatide-treated rats still displayed a profound reduction in gastric emptying at the 14-day time-point [35]. The data suggest that the "gastric inhibitory" GLP-1 receptors in rats are subject to desensitization-tachyphylaxis but this effect seems to be dependent on full 24-h exposure as obtained by liraglutide.

Other studies showed equivalence in gastric emptying between liraglutide and placebo over the full postprandial period after short-term treatment [36], but slower gastric emptying with liraglutide during the initial hour [37].

In our study we demonstrated that there is no difference between the two treatments on postprandial lipaemia both on first administration and after a two-week treatment. These findings suggest that there are mechanisms beyond the inhibition of gastric emptying that participate in the regulation of lipid postprandial metabolism following the treatment with liraglutide or exenatide.

We are well aware of the limitations of our study, which we consider to be the small number of participants and its short duration. However, we consider that the similarity of the two treatment groups in terms of baseline characteristics is an important advantage.

In conclusion, exenatide and liraglutide both appear to be equally effective in lowering postprandial lipaemia after the first administration and after a two-week treatment. Further investigation might be needed to elucidate the mechanisms which lead to this phenomenon, which seem to be independent of gastric emptying.

Conflict of Interests

The authors declare that there is no conflict of interests regarding to the publication of this paper.

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Research Article

Relationship between Autonomic Nervous System Function and Continuous Interstitial Glucose Measurement in Patients with Type 2 Diabetes

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Aims. The Aim of the present study was to examine whether there is a relationship between autonomic nervous system function and glycemic variability (GV) in patients with type 2 diabetes (T2D). *Methods*. A total of 50 (29 males) patients with T2D (mean age 58.4 ± 9.9 years, median diabetes duration 5.5 [IQR 2.0–9.25] years), on oral antidiabetic agents, underwent ECG recording and subcutaneous glucose monitoring, simultaneously and continuously, for 24 hours. *Results*. After adjustment for HbA1c and diabetes duration, total power of heart rate variability (HRV) was inversely associated with the standard deviation of the mean interstitial tissue glucose (MITG) and with the M-value during the entire recording (r: −0.29, $P = 0.052$; r: −0.30, $P = 0.047$, resp.) and during the night (r: −0.29, $P = 0.047$; r: −0.31, $P = 0.03$, resp.). Most of the HRV time-domain indices were significantly correlated with standard deviation of the MITG and the M-value. These correlations were stronger for the HRV recordings during the night. No significant association was found between HRV parameters and MAGE. *Conclusions*. HRV is inversely associated with GV in patients with T2D, which might be a sign of causation between GV and autonomic dysfunction. Prospective studies are needed to further investigate the importance of GV in the pathogenesis of long-term complications of diabetes.

1. Introduction

In normal individuals the heart rate has a high degree of beat-to-beat variability, fluctuating with respiration, increasing with inspiration and decreasing with expiration. Cardiac autonomic neuropathy (CAN) in diabetes results from damage to the autonomic nerve fibers (parasympathetic and sympathetic) that innervate the heart and blood vessels, with resultant abnormalities in heart rate control and vascular dynamics [1], ultimately leading to diminished heart rate variability (HRV) [2]. Clinical symptoms of autonomic dysfunction may not appear until long after diabetes onset, but subclinical CAN, manifested by reduced HRV (the earliest indicator of CAN), may be detected as early as within 1-2 years after diagnosis of diabetes [3].

CAN is an often overlooked complication of diabetes [4], associated with dire consequences regarding morbidity and mortality [5]. It carries a high risk of cardiac arrhythmias and sudden death, possibly related to silent myocardial ischemia [6]. There is no widely accepted single approach to the diagnosis of CAN in diabetes. Assessment of heart rate variability (HRV), orthostatic hypotension, and 24 h blood pressure profiles provide indices of both parasympathetic and sympathetic autonomic function and can be used in clinical settings. Specifically, HRV provides a noninvasive and objective method for assessing cardiovagal function and may

be derived from electrocardiogram recordings [7]. Twenty-four-hour ECG recordings allow calculation of more complex statistical time-domain measures and together with spectral analysis of HRV allow for a better delineation of cardiac autonomic function [8].

The concept of glycemic variability (GV) in type 2 diabetes (T2D) has recently attracted great interest for several reasons, including association with higher levels of markers of oxidative stress [9] and increased mortality in certain circumstances, such as sepsis and critical illness [10]. Despite experimental evidence, however, in clinical ground, there is insufficient evidence to support an independent relationship between blood glucose fluctuations and long-term complications of diabetes; the issue remains to be controversial [11]. So far it is not known whether glycemic variability is associated with cardiac autonomic dysfunction in diabetic patients [12]. It is intriguing to hypothesize that increased glycemic variability may lead to autonomic imbalance through oxidative stress and acute elevation of proinflammatory cytokines [13]. Glycemic variability can be assessed more precisely nowadays with the use of special devices [14] that measure the interstitial tissue glucose levels continuously over a period of several days (continuous glucose monitoring systems [CGMS]) [15].

The aim of the present cross-sectional study was to investigate the relationship between cardiac autonomic function (evaluated by HRV during continuous ECG recording) and GV (assessed by simultaneous continuous interstitial tissue glucose monitoring) in patients with type 2 diabetes.

2. Materials and Methods

2.1. Study Sample and Standard Procedures. The study population included 50 (29 males) patients with T2D (mean age 58.4 ± 9.9 years, median diabetes duration 5.5 [IQR 2.0–9.25] years), treated with oral antidiabetic agents. The patients who took part in the study were attending the outpatient diabetes clinic of the Laiko General Hospital in Athens, Greece. Patients receiving insulin or medications affecting cardiac rate and patients with uncontrolled thyroid disease, alcoholism, or any acute illness were excluded. All patients had a complete screening history and physical examination before inclusion into the study, to assess for the presence of microvascular (retinopathy, nephropathy, and neuropathy) or macrovascular complications.

Participants presented to the diabetes research clinic in the morning, after an overnight fast. They had been asked to abstain from alcohol and smoking for at least 12 hours before presentation and not to take their glucose-lowering and antihypertensive therapy (if any) on the morning of each visit.

Body weight and height were measured in light clothing and body mass index (BMI, the ratio of weight [in kg] to height [in m^2]) was calculated. Brachial blood pressure (BP) was measured using a semiautomatic BP device with the subjects rested for 5 minutes. Blood samples were obtained in order to measure fasting plasma lipids (total cholesterol, triglycerides, HDL-cholesterol, and LDL-cholesterol), as well as glucose and HbA1c. LDL-cholesterol was calculated using the Friedewald formula [16].

The study was carried out in accordance with the principles of the Declaration of Helsinki as revised in 2008 [17] and was approved by the participating hospital's ethics committee. Written informed consent was obtained from all participants.

2.2. Evaluation of Autonomic Function. All patients underwent continuous ECG Holter monitoring for 24 hours. For this study the digital ECG Holter recorder Spider View (ELA Medical, France) with seven electrodes was used to record three-channel ECGs. The 24-hour recordings were analysed using the SyneScope Holter analysis software (version 3.00 ELA Medical, France). Artefacts and ectopic beats were automatically excluded from analysis. In addition, the QRS complex classification was reviewed by an experienced cardiologist blinded to the patients' clinical characteristics. All of the HRV time- and frequency-domain parameters, recommended by the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, were calculated based on the 24-hour recordings [7]. The values of time-domain parameters were expressed in milliseconds (ms). HRV in the frequency-domain was computed by SyneScope using fast Fourier transformation analysis. The total power and, respectively, the power in very low frequency (VLF, ≤0.04 Hz), low frequency (LF, 0.04–0.15 Hz), and high frequency (HF, 0.15–0.40 Hz) bands were measured. All the frequency-domain parameters of HRV were calculated as absolute values and expressed in ms^2. Furthermore, the LF and HF powers were expressed in normalised units (nu), representing the relative value of each power component in proportion to the total power minus the VLF component [7].

2.3. Evaluation of Glucose Variability. A commercially available device based on the microdialysis technique was used for the continuous interstitial glucose monitoring (GlucoDay, Menarini Diagnostics). A microdialysis fiber was inserted by a medical professional and calibrated with a blood glucose test; glucose results were stored for retrospective analysis for 24 h, acquiring data every 5 min (288 measurements/day), after which the fiber was removed [14]. The CGMS was calibrated in a standard way as recommended by the manufacturer, using four validated self-monitored blood glucose levels during the 24 h monitoring period [18]. During this period, the patients were asked to have normal activities at home and at work and were advised to take their medications as usual. The following indices of glycemic variability were calculated from the stored glucose data:

(a) the standard deviation of the mean glucose value (SDMG), a measure of the dispersion of all the blood glucose values;

(b) the mean amplitude of glycemic excursions (MAGE) calculated by taking the arithmetic mean of the interstitial tissue glucose increases or decreases (from nadirs to peaks or vice versa) provided that the differences are greater than one SD of the mean glucose value [19];

(c) the M-value, a logarithmic transformation of the deviation of glycemia from an arbitrary assigned "ideal" glucose value (6.67 mmol/L [120 mg/dl]) [20].

2.4. Statistical Analysis. Normally distributed variables are presented as mean ± SD, while the median (25th–75th, interquartile range) is used for variables with a skewed distribution. Continuous variables were tested for normal distribution by the Kolmogorov-Smirnov test. Data not normally distributed were log-transformed for analysis. Hence, all HRV and GV indices were log-transformed due to their skewed distribution. Pearson's correlation coefficient was used to investigate associations between variables. Partial correlation was used to control for the effect of other, possibly confounding, variables. All reported P values are from two-sided tests and compared to a significance level of 5%. Data were analyzed using the Statistical Package SPSS, version 19.0 (SPSS Inc., Chicago, IL, USA).

3. Results

The demographic and clinical characteristics of the patients are shown in Table 1. They had relatively good glycemic control, as evidenced by their adequate HbA1c levels. The great majority (94%) were being treated with one or more glucose-lowering medications, mostly metformin (52%), sulphonylureas, (20%), and sitagliptin (16%), in various combinations. The mean BMI was 30.8 ± 5.7 kg/m^2. More than half of the patients (62%) had been diagnosed with arterial hypertension and 42% were smokers.

The glycemic indices derived from CGMS are presented in Table 2. Mean interstitial tissue glucose (MITG) had a positive correlation with HbA1c (r: 0.55, $P < 0.001$), while the GV indices were also significantly (but weakly) positively correlated with HbA1c (MAGE: r: 0.32, $P = 0.02$; SDMG: r: 0.31, $P = 0.03$; M-value: r: 0.27, $P = 0.059$) and with diabetes duration (MAGE: r: 0.32 $P = 0.02$; SDMG: r: 0.36, $P = 0.01$; M-value: r: 0.29, $P = 0.04$).

Total power of HRV was negatively correlated with HbA1c (r: −0.32, $P = 0.024$) but not with age, BMI, diabetes duration, systolic and diastolic BP, smoking, or lipid parameters. The correlation with HbA1c was stronger during the day (r: −0.38, $P = 0.009$) than during the night (r: −0.31, $P = 0.03$). Of the other HRV frequency-domain parameters, HF, but not LF, was correlated with HbA1c in a similar pattern as total power, while the ratio of LF/HF was positively correlated with HbA1c (r: 0.29, $P = 0.04$). Concerning the time-domain parameters of HRV, HbA1c was significantly correlated with SDNN and RMSSD, only during the night (r: −0.32, $P = 0.02$ and r: −0.36, $P = 0.02$, resp.). Total power of HRV was inversely and significantly associated with MITG (r: −0.36, $P = 0.01$). The other parameters of frequency-domain analysis were not significantly related to MITG. Most time domain parameters of HRV were inversely associated with MITG, but this correlation concerns only HRV parameters at night period. All associations, however, between HRV parameters and MITG disappeared after adjustment for HbA1c and duration of diabetes (Tables 3 and 4).

Table 1: Patients' demographic and clinical characteristics.

Variable	Value
N	50
Gender [male n (%)]	29 (58)
Age (years)	58.4 ± 9.9
BMI (kg/m^2)	30.8 ± 5.7
Systolic blood pressure (mm Hg)	128.3 ± 15.5
Diastolic blood pressure (mm Hg)	74.9 ± 10.2
Fasting glucose (mmol/L)	7.7 ± 2.2
Total cholesterol (mmol/L)	4.7 ± 0.9
Triglycerides (mmol/L)*	1.6 (1.2–2.2)
HDL-C (mmol/L)	1.2 ± 0.2
LDL-C (mmol/L)	2.8 ± 0.9
Fasting glucose (mmol/L)	7.7 ± 2.2
HbA1c (mmol/mol)	54.3 ± 12.6
Duration of diabetes (years)*	5.5 (2.0–9.25)
Presence of hypertension	31 (62)
Smokers	21 (42)
Treatment with sulphonylureas	26 (52)
Treatment with metformin	47 (94)
Treatment with meglitinides	1 (2)
Treatment with thiazolidinediones	8 (16)
Treatment with sitagliptin	10 (20)

(Data are presented as mean (±SD) or as n (%).
*Median value (interquartile range).
BMI: body mass index, HDL-C: high density lipoprotein cholesterol, and LDL-C: low density lipoprotein cholesterol.
Hypertension was defined as systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg or the use of antihypertensive medication.

Table 2: Indices related to glycemia as obtained from the continuous glucose monitoring system (CGMS).

Variable	Value
Mean interstitial tissue glucose (mmol/L)	7.9 ± 1.7
SD of mean glucose* (mmol/L)	2.13 (1.76–0.05)
M-value*	55.1 (37.67–119.85)
MAGE*	92.0 (75.20–121.20)

Data are expressed as mean ± SD.
*Median value (interquartile range).
MAGE: mean amplitude of glycemic excursions.

There was an overall inverse correlation between both frequency- and time-domain parameters of HRV and indices of GV. In univariable analysis, the correlation between total power and SDMG was significant (r: −0.30, $P = 0.03$) and stronger for the night component (r: −0.32, $P = 0.02$). Total power was also significantly correlated with M-value (r: −0.34, $P = 0.02$ for the total time recording and r: −0.36, $P = 0.01$ for the night component). The remaining parameters of frequency-domain analysis (LF, HF and LF/HF) were not significantly correlated with GV, while the time-domain indices (SDNN, PNN30, PNN50 and SDANN) were significantly correlated with SDMG and M-value. The above significant correlations were stronger for the night component of all recordings. There was no statistically significant

Table 3: Correlation analysis between frequency-domain parameters of HRV, mean interstitial tissue glucose, and indices of glycemic variability.

Variable	Mean interstitial tissue glucose		MAGE		SD of mean glucose		*M*-value	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
TP (t)	−0.25	0.09	−0.14	0.37	**−0.29**	**0.052**	**−0.30**	**0.047**
TP (n)	−0.25	0.09	−0.13	0.39	**−0.29**	**0.047**	**−0.31**	**0.034**
LF (t)	−0.19	0.22	−0.03	0.82	−0.14	0.35	−0.13	0.38
LF (n)	−0.20	0.19	−0.02	0.90	−0.14	0.36	−0.14	0.35
HF (t)	−0.08	0.59	0.05	0.76	−0.10	0.51	−0.16	0.29
HF (n)	−0.09	0.57	0.07	0.63	−0.09	0.56	−0.16	0.28
LF/HF (t)	−0.13	0.37	−0.11	0.46	−0.05	0.76	0.05	0.73
LF/HF (n)	−0.11	0.48	−0.12	0.44	−0.04	0.81	0.07	0.67

Data are adjusted for HbA1c and duration of diabetes.
All variables are log-transformed due to skewness.
(t): Total; (n): night
Night period: 23:00–06:00
TP: Variance of all normal-to-normal NN intervals
LF: Power in the low frequency range
HF: Power in the high frequency range
LF/HF: Ratio LF/HF.

Table 4: Correlation analysis between time-domain parameters of HRV, mean interstitial tissue glucose, and indices of glycemic variability.

Variable	Mean interstitial tissue glucose		MAGE		SD of mean glucose		*M*-value	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
SDRR (t)	−0.29	0.052	−0.06	0.70	−0.23	0.13	**−0.28**	**0.06**
SDRR (n)	−0.28	0.058	−0.19	0.20	**−0.39**	**0.007**	**−0.40**	**0.006**
PNN30 (t)	−0.07	0.64	−0.02	0.87	−0.15	0.32	−0.25	0.09
PNN30 (n)	−0.14	0.36	−0.06	0.72	−0.19	0.22	−0.27	0.07
PNN50 (t)	−0.11	0.47	−0.06	0.72	−0.14	0.36	−0.27	0.07
PNN50 (n)	−0.20	0.19	−0.10	0.49	**−0.23**	**0.12**	**−0.35**	**0.02**
SDANN (t)	−0.29	0.054	−0.04	0.79	−0.21	0.16	−0.27	0.07
SDANN (n)	−0.25	0.09	−0.18	0.22	**−0.38**	**0.009**	**−0.37**	**0.01**
RMMSD (t)	0.05	0.75	0.03	0.86	−0.04	0.80	−0.14	0.36
RMSSD (n)	−0.01	0.93	0.02	0.87	−0.08	0.62	−0.18	0.24

Data are adjusted for HbA1c and duration of diabetes.
All variables are log-transformed due to skewness.
(t): Total; (n): night.
Night period: 23:00–06:00
SDRR: Standard deviation of all normal-to-normal RR intervals
PNN30: Number of pairs of adjacent NN intervals differing >30 ms in the entire recording divided by the total number of all NN intervals
PNN50: Number of pairs of adjacent NN intervals differing >50 ms in the entire recording divided by the total number of all NN intervals
SDANN: 5 min, standard deviation of the changes of NN intervals in all 5-minute segments of the entire recording
RMSSD: The square root of the mean of the sum of the squares of differences between adjacent NN intervals.

association between MAGE and indices of HRV. The relation between HRV parameters and GV was further evaluated after adjustment for HbA1c and diabetes duration, resulting in slight attenuation but remaining, in most cases, statistically significant (Tables 3 and 4).

4. Discussion

In the present study, it was shown for the first time that GV, calculated during a 24 h CGMS recording, is independently associated with autonomic nervous system function, assessed by simultaneous analysis of HRV, in patients with type 2 diabetes. To our knowledge, only one previous study has investigated HRV and GV in parallel, by simultaneous ECG and CGMS recordings, in patients with type 2 diabetes [21]. However, in that study, only a positive association (r: 0.40, $P = 0.04$) between the ratio of LF/HF and MAGE was reported.

The association between HRV and glycemia in the present study has been observed at three levels. At the first level, total power, a measure of total variance of HRV, and HF, a measure of vagal activity [7], were inversely associated with HbA1c, a measure of average glycemia during the three months preceding the experiment. At the second level, total power was inversely associated with MITG, a measure of glycemic exposure during the experiment. Interestingly, MITG was also inversely associated with SDNN and SDANN, both indices of overall variability in time-domain analysis

of HRV recordings [7]. At the third level, it was shown that overall heart rate variance, expressed by the total power in frequency-domain analysis and by SDNN, PNN50, and SDANN in time-domain analysis, was inversely, significantly, and independently associated with GV, expressed by M-value and (partly) by SDMG, but not by MAGE.

The inverse association between HRV and chronic hyperglycemia has been previously shown in several studies in patients with and without diabetes and there is strong evidence suggesting that chronic hyperglycemia is involved in the development of autonomic neural imbalance [2, 22, 23]. The presence of reduced HRV in patients with diabetes has been attributed to cardiac autonomic impairment, which appears to be present at early stages of diabetic metabolic impairment [23, 24]. The association, however, between HRV and simultaneously/continuously recorded glycemia has been investigated to a much lesser extent. To our knowledge, in the only previous published study where simultaneous HRV and glucose readings were examined, the relation between HRV indices and mean glucose levels was not reported [21]. In the present study, indices of HRV were inversely related with MITG, but the associations were not statistically significant after adjustment for HbA1c and duration of diabetes. On the contrary, the association between certain indices of HRV (total power, SDANN, SDNN, and PNN50) and GV were sustained after such adjustment (Tables 3 and 4).

The association between GV and autonomic function in the present study should be interpreted with caution. The cross-sectional design does not allow for causal relationships to be inferred. A possible association between GV and autonomic dysfunction has been suggested on the basis of a hypothesized broader association between GV and the development of chronic diabetic complications. Although the role of chronic hyperglycemia in the development of small/large vessel and nerve damage is well established [25], whether or not GV is independently involved in the pathogenesis of microvascular and macrovascular diabetic complications is an issue of debate [11]. In a separate analysis of DCCT data, Kilpatrick et al. [26] showed that the variability in blood glucose around a patient's mean value has no influence on the development or progression of either retinopathy or nephropathy in type 1 diabetes. Another analysis of the DCCT data [27], however, showed that increasing variability in HbA1c adds to the risk of microvascular complications, implying a significant role of longer-term glucose variability in the development of retinopathy and nephropathy in type 1 diabetes, in contrast to the effect of short-term glucose variability on complication risk.

The inverse HRV/GV association in the present study could also be attributed to a short-term influence of acute glucose elevations on sympathetic activity. It has been repeatedly shown that both hyperglycemia and hyperinsulinemia produce sympathetic activation in normal individuals [28], while the effect of glucose peaks on sympathetic function in patients with diabetes is not known. On the other hand, it is well known that hypoglycemia stimulates an acute adrenergic response both in normal individuals and in patients with diabetes [29]. Furthermore, this association might be looked at from the opposite angle. It might be thus hypothesized that autonomic nervous system dysfunction could result in larger fluctuations in blood glucose, due to disturbed gastrointestinal tract motility or an impaired counterregulatory response to low glycemic levels [30]. However, the short duration of diabetes, the absence of other complications, and the relatively high mean HRV indices of the study population render this possibility rather unlikely.

The association between HRV and GV was observed only for certain indices. Hence, in frequency-domain analysis, only total power was significantly related to GV, while most time-domain indices had a significant association with GV, especially during the night. It should be noted that the interpretation of frequency-domain analysis calculations during long-term recordings is not well defined [7]. Physiological mechanisms of heart period modulations are not stationary during the 24-hour period; hence the physiological interpretation of the spectral components (VLF, LF, and HF power components) calculated over 24 hours is difficult. On the other hand, time-domain indices are more reliable measures of overall heart rate variance on the long term [7].

The GV indices associated with HRV included SD of mean glucose and M-value, while no association was observed between MAGE and HRV. Although there is no "gold standard" for determining glucose variability, according to a recent review [31] SDMG might be the preferable method when quantifying variability from CGMS data, because it is the easiest and best validated measure. In the same review it is suggested that M-value is rather a clinical than a mathematical indicator of glycemic control, because hypoglycemia has a greater impact on it than hyperglycemia [31]. Furthermore, MAGE arbitrarily ignores glucose excursions of less than 1 SD [19], a fact that may incorrectly disregard possibly important smaller excursions [31]. As the population of the present study had a relatively short diabetes duration and good glycemic control, it is possible that glycemic fluctuations were not sufficiently captured by MAGE.

The main strength of the present study is that the investigation of HRV and GV has been performed simultaneously during 24 hours. Furthermore, the study population consisted of patients with early-stage type 2 diabetes and good glycemic control, allowing for detection of early disturbances in HRV with relatively modest fluctuations in glucose levels. In addition, the latter was measured with CGMS, in order to capture variations in interstitial tissue glucose levels as frequently as every 5 minutes.

The main limitation of the study is that its cross-sectional design does not allow for causal relationships to be identified. Hence, the observed associations may only serve as hypothesis generating.

In conclusion, the present study showed that HRV is associated with GV in patients with type 2 diabetes with a relatively short course of disease. This association might be a sign of causation between GV and diabetic complications, although other explanations could also apply. Prospective studies are needed to further investigate the importance of GV in the pathogenesis of autonomic dysfunction and other long-term complications of diabetes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Real Life Cancer Comorbidity in Greek Patients with Diabetes Mellitus Followed Up at a Single Diabetes Center: An Unappreciated New Diabetes Complication

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We determined cancer comorbidity in patients with diabetes followed up at a single Greek academic clinic and investigated the potential related factors. Cancer comorbidity was prospectively recorded for all patients with type 2 (T2DM, $n = 759$) or type 1 (T1DM, $n = 134$) diabetes of at least 10-year duration examined during one year. Patient characteristics, diabetes age of onset, duration, treatment, control, and complication rates were compared between subjects with and without cancer. Moreover, a retrospective collection of data from similar patients examined for the first time during the last 25 years, but lost to follow-up, after at least one-year's regular visits, was performed. In regularly followed-up T2DM patients cancer comorbidity was 12.6%. Patients with cancer were older and more frequently smokers. Prostate cancer was the most frequent (24.0%) type. In T1DM cancer comorbidity was 3.0%. Similar rates of comorbidity and types of cancer were observed in lost to follow-up patients. In conclusion, our patients with T2DM of at least 10-year' duration show high cancer comorbidity. No specific characteristics discriminate patients with cancer. Therefore presymptomatic cancer detection and prevention strategies may have to be incorporated into the annual systematic evaluation of our patients.

1. Introduction

Type 2 diabetes (T2DM) mellitus is highly prevalent and tends to become a "global epidemic." The increase in T2DM incidence is in parallel to the "twin" "global pandemic" of obesity and is mainly attributed to it [1]. Both obesity and T2DM are independently associated with an increased risk of developing cancer and an increased mortality [2, 3]. In the interplay between obesity, T2DM, and cancer, insulin resistance and hyperinsulinemia are important determinants [4]. Insulin and insulin-like growth factors are well known as key regulators of metabolism and growth, affecting cell proliferation, differentiation, and apoptosis, mechanisms via which they are thought to be involved in the oncogenesis process [5]. Both T2DM and cancer have been associated with exposure to common environmental factors, such as high consumption of energy and animal fat and low consumption of fiber as well as low physical activity. These environmental factors are involved in the development of insulin resistance and compensatory hyperinsulinemia, and thus they influence the risk for both diabetes and cancer evolution [6].

The relation between T2DM and malignancies has been mostly studied in the form of the comparison of cancer development risk between subjects with diabetes and without diabetes. Subjects with type 2 diabetes mellitus (T2DM) have been found at an increased risk of cancer development compared to subjects without diabetes, even after adjustment for age, BMI, smoking, nutritional habits, and physical activity [2, 7].

Unlike T2DM, studies reporting on the association between type 1 diabetes (T1DM) and cancer risk are scanty. T1DM has been associated with a modest excess cancer risk overall and risks of specific cancers that differ from those associated with T2DM. In a Swedish study, patients with T1DM had elevated risks of cancers of the stomach, cervix, and endometrium [8]. In another study, ovarian cancer risk

was found to be highly significantly raised in patients with T1DM diagnosed at ages 10–19 years [9].

Unfortunately, in Greece no reliable data on cancer incidence exist and, therefore, cancer morbidity cannot be easily compared between Greek subjects with or without diabetes.

During regular follow-up in the outpatient diabetes clinic, the authors had the impression that the frequency of malignancies appeared very high. Therefore, the present study was conducted to ascertain cancer comorbidity in our patients and to investigate potential related factors.

2. Patients and Methods

Data of all patients older than 20 years of age, having T2DM or T1DM of at least 10-year duration, was prospectively collected during their regular follow-up visits during one-year period. This relatively long diabetes duration was selected in order to ensure adequate exposure of the subjects to the diabetic milieu. The study was approved by the institutional ethics committee (number of approval ES 10/29) and had no funding.

Overall, 759 subjects with T2DM and 134 subjects with T1DM were included.Cancer comorbidity was recorded, as well as the type of cancer and the year of its diagnosis. Patients with diabetes onset after cancer diagnosis, as well as patients with drug-induced diabetes, were excluded. Patients with T2DM and T1DM were analyzed separately. Sex, age, age of diabetes onset, diabetes duration, anthropometrical data (body weight, height, BMI, and waist circumference), blood pressure levels, smoking status, antidiabetic medication, glycemic control, and frequency of diabetes complications were studied.

After analysis of data from regularly followed-up subjects, we also performed a retrospective collection of data from patients with the same characteristics, examined for the first time in our outpatient clinic during the last 25 years, but lost to follow-up after at least one-year regular visits. Cancer comorbidity, type of cancer, and year of cancer diagnosis were recorded wherever information was available. This was done in order to assess the already known cancer comorbidity. Naturally, no comparisons between subjects with or without cancer could be conducted in this cohort, since the real cancer comorbidity in subjects lost to follow-up might have been higher, due to cancer development after the patients' last visit to the clinic.

Statistical analysis was carried out by the statistical package for social sciences (SPSS, Chicago, IL) version 15. Differences between subjects with and without cancer were evaluated with Student's t-test for continuous variables with normal distribution and with x^2 test for categorical variables. A p correction for multiple comparisons was performed. Nonparametric tests were used where appropriate.

3. Results

Mean age of T2DM patients was 68.7 years, mean diabetes duration 19.6 years, mean BMI 28.5 kg/m^2, and mean HbA_{1c} 7.0%. The corresponding values for T1DM patients were age of 46.5 years, mean diabetes duration of 23.1 years, mean BMI of 25.6 kg/m^2, and mean HbA_{1c} of 7.5%. Most of the T2DM patients were on oral antidiabetic drugs (OADs, 59.2%), while 9.3% were treated with OADs plus basal insulin and 31.5% were exclusively treated with insulin. Current smokers or exsmokers were 52.7% of T2DM and 47.9% of T1DM patients. Cancer comorbidity was observed in 12.6% of T2DM patients. Mean age of cancer diagnosis was 65.2 ± 1.1 and cancer was diagnosed after 14.0 ± 1.0 years of diabetes duration. On the other hand, cancer comorbidity was observed in 3.0% of T1DM patients. The mean age of cancer development in T1DM patients was 46.5 ± 9.5 years.

The characteristics of patients with cancer in comparison to those without cancer are shown in Table 1. In T2DM subjects, patients with cancer were older, were diagnosed with diabetes at an older age, and were more frequently present or past smokers compared to patients without cancer. They did not differ in sex, anthropometric measurements, blood pressure levels, glycemic control, antidiabetic regime, duration of insulin therapy, or coexistence of diabetes complications. No difference in cancer comorbidity was observed between patients who developed T2DM before or after their 50th year of age or between patients who had T2DM duration lower or greater than 20 years. Similarly, no difference in cancer comorbidity was observed between normal weight, overweight, or obese T2DM patients (data not shown). Finally, no difference in cancer comorbidity was observed between patients taking or not taking metformin and patients on insulin therapy (including insulin glargine) compared to patients not on insulin therapy.

Cancer types in subjects with T2DM regularly followed up are shown in Table 2. Prostate cancer was the most frequently observed type of cancer in our patients (24.0%), followed by breast cancer, and so forth. Surprisingly, colon cancer was not common in our patients. The same was true for lung cancer, despite the high number of present smokers and exsmokers. In T1DM patients there were two breast cancers, one astrocytoma, and one case of Hodgkin's disease. All subjects developed cancer before the 14th year of diabetes duration.

After finding this considerably high comorbidity of cancer in our systematically followed-up patients, we retrospectively examined which was the rate of known cancer, in other patients followed up in our center in the past, but lost to follow up at present. These patients were examined for the first time in our outpatient clinic during the last 25 years and in order to be selected they had to have at least 10 years diabetes dutration. There were 950 T2DM patients with a cancer comorbidity of 10.6% and a mean age of cancer development of 65.7 ± 0.1 years and 102 T1DM patients with a cancer comorbidity of 2.0% (referring only to breast cancer) and a mean age of cancer development of 56.5±10.5 years. The lost to follow-up patients' characteristics are shown in Table 3. The types of cancer in T2DM patients lost to follow-up are shown in Table 4.

They had mean diabetes duration (18.0 ± 0.2 years), BMI (27.9 ± 0.1 kg/m^2), and HbA_{1c} 7.5 ± 0.0%, very similar to regularly followed-up subjects. Moreover, most of them were also on OADs (68.6%), while 2.3% were on OADs plus basal

Table 1: Characteristics of regularly followed-up subjects with cancer in comparison to subjects without cancer.

	T2DM			T1DM		
	CA + (n = 96) 12.6%	CA − (n = 663)	P	CA + (n = 4) 3.0%	CA − (n = 127)	P
Sex (males)	63.5%	57.9%	NS	50.0%	50.0%	NS
Age	70.9 ± 1.0	68.3 ± 0.4	<0.05	49.0 ± 9.0	46.4 ± 1.3	NS
Age of diabetes onset	51.2 ± 1.1	48.9 ± 0.4	<0.05	37.7 ± 8.7	23.4 ± 1.2	<0.05
Diabetes duration (years)	20.8 ± 0.9	19.5 ± 0.3	NS	11.5 ± 0.6	23.5 ± 0.9	<0.05
Smoking	66.7%	50.8%	<0.01	50.0%	47.9%	NS
BMI	28.5 ± 0.4	28.5 ± 0.2	NS	30.2 ± 2.2	25.5 ± 0.3	NS
Weight (kg) (males)	79.9 ± 1.5	80.1 ± 0.6	NS	95.5 ± 7.5	80.0 ± 1.3	NS
Weight (kg) (females)	75.8 ± 1.7	73.7 ± 0.9	NS	75.0 ± 18.0	65.3 ± 1.4	NS
Height (m) (males)	1.70 ± 0.0	1.70 ± 0.0	NS	1.83 ± 0.1	1.80 ± 0.0	NS
Height (m) (females)	1.58 ± 0.0	1.58 ± 0.0	NS	1.52 ± 0.0	1.60 ± 0.0	NS
Waist (cm) (males)	99.8 ± 1.2	99.1 ± 0.9	NS	101.2 ± 5.1	98.9 ± 1.3	NS
Waist (cm) (females)	95.8 ± 1.6	96.5 ± 0.3	NS	97.9 ± 1.1	87.6 ± 1.1	NS
SAP (mmHg)	138.2 ± 1.4	133.6 ± 1.5	NS	130.3 ± 1.0	129.6 ± 1.2	NS
DAP (mmHg)	79.6 ± 1.2	74.8 ± 0.8	NS	73.2 ± 0.2	75.8 ± 0.6	NS
A1c%	7.0 ± 0.1	7.0 ± 0.0	NS	8.1 ± 0.4	7.5 ± 0.4	NS
OADs (%)	53.1%	60.0%	NS			
OADs + basal insulin (%)	13.5%	8.7%	NS			
Insulin treated (%)	33.3%	31.2%	NS			
Years of diabetes before insulin initiation	16.3 ± 1.2	14.9 ± 0.5	NS			
Duration of insulin therapy (years)	7.2 ± 1.0	7.2 ± 0.4	NS			
"Classical" diabetes complications coexistence (%)	57.1%	68.8%	NS	66.7%	47.4%	NS

OADs: oral antidiabetic drugs.

insulin and 29.1% were exclusively insulin treated. Prostate cancer was the most frequently observed type (14.9%), as it was for the regularly followed-up patients, followed by breast, urinary tract, and colon cancer (12.9% for each), hematological malignancies, and so forth.

4. Discussion

The present study is the first one, to the best of our knowledge, to outline the "real life" problem of cancer comorbidity among systematically followed-up patients in a single outpatient diabetes clinic in Greece. Patients with at least 10 years of diabetes duration were selected, in order to have adequate exposure to the diabetic state and thus exhibit possible effect of diabetes on oncogenicity, which is well known to be a long-lasting procedure. Some studies have shown an association between newly diagnosed diabetes and especially pancreatic cancer [10], but, in that case, diabetes should be considered a result, that is, a clinical manifestation of cancer, than a causal factor.

Cancer comorbidity of 12.6% in regularly followed-up and of 10.6% in lost to follow-up T2DM patients was observed in our study. Moreover much lower cancer comorbidity (3.0% in regularly followed-up and 2.0% in lost to follow-up patients) was observed in T1DM patients, who were of course much younger. Unfortunately data on cancer prevalence are lacking in Greece, so the cancer comorbidity of our study population cannot be compared with that of the general population. By any means, this was not the aim of our study, since the knowledge that cancer is more prevalent in subjects with diabetes seems to be supported by several lines of evidence.

Patients with diabetes are an extremely heterogeneous group in terms of glycemic control, coexistence of diabetes complications, medication used, diet, degree of obesity, and endogenous insulin levels.Systemically followed-up patients in the same center are of course individually treated but, more or less, treated according to the same therapeutic perception. This diminishes discrepancies in the clinical course of the patients caused by exogenous factors and whatever happens to them can only be attributed to each individual patient's particularities (biological, etc.). In the present study no difference in metformin or insulin use was observed between patients with or without cancer.

In the present study, no difference in BMI was observed between subjects with and without cancer, both for T1DM and T2DM. Moreover, no difference in cancer comorbidity was observed between normal weight, overweight, and obese T2DM subjects. These findings, merely referred to in the context of the results of the present study, do not seem to have a special interpretation and should not take anyone's thought

Table 2: Types of cancer in subjects with T2DM regularly followed up.

	n	%
Urinary tract		
Prostate	23	24.0
Bladder	7	7.3
Kidney	2	2.1
Breast		
Breast	14	14.6
Hematological malignancies		
Lymphomas	6	6.3
Leukemias	3	3.0
Gastrointestinal		
Colon	8	8.3
Pancreas	2	2.1
Stomach	1	1.0
HCC	1	1.0
Cholangiocarcinoma	1	1.0
GIST	1	1.0
Lung		
Lung	5	5.2
Female genitals		
Endometrial	3	3.1
Ovarian	3	3.1
Thyroid		
Thyroid	6	6.3
Skin		
Nonmelanoma	3	3.1
Melanoma	2	2.1
Rest		
Larynx	2	2.1
Pharynx	1	1.0
Buccal	1	1.0
Chondrosarcoma	1	1.0
Total	**96**	**100.0**

off the fact that being overweight is second only to smoking as a clear and avoidable cause of cancer [11].

The association between smoking and the development of cancer in general and especially of some types of cancer is well documented [12]. The same is true for the association between smoking and cardiovascular disease [13]. This is the reason why smoking cessation is promoted and instructed with emphasis in our center in all smoking patients, at every visit. Nevertheless, many of our patients are current smokers or exsmokers. As it can be presumed, the percentage of smokers was significantly higher in T2DM patients with cancer in comparison to those without cancer. These two facts testify the urgent need to persuade and help patients quit smoking.

HbA_{1c} levels and the presence of the classical diabetes complications did not differ between subjects with or without cancer, at least in our population, showing possibly that cancer development in patients with diabetes is not associated with the severity of diabetes, but with something else, such as the already known common for the two conditions risk factors or something unknown till present. Of course one must not forget that every unexplained worsening of diabetes control might be an alert signal for cancer development.

The most common type of malignancy in our patients was prostate cancer, although in the literature diabetes is associated with reduced risk of prostate cancer [14], mainly due to lower serum testosterone concentration found in diabetic men compared to nondiabetic [15]. The findings of our study are in line with those of Veterans Integrated Services Network study, where, in 87,678 male patients with diabetes, cancer comorbidity was 11.3% in total, almost as it was in our study, and prostate cancer was the most frequently observed type [16].

Breast cancer, which was the second cancer in frequency in T2DM patients and the most frequent cancer in T1DM patients in our study, is referred in most but not all studies to be associated with diabetes, especially in postmenopausal women, with a varying relative risk in different studies and a 1.20 relative risk found in a meta-analysis [17].

Colon cancer was not common in our patients (8.3%). Colorectal carcinogenesis is a model of diabetes related oncogenesis and is associated with hyperinsulinemia. Both diabetes and colon cancer development have been attributed to exposure to common risk factors such as energy dense, high fat, low fibre diets, and physical inactivity [18].

Lung cancer, the most common cancer in Southern Europe, was not frequent in our patients (5.2%), despite the great number of present smokers and exsmokers. A study based on Great Britain's General Practice Research Database, with more than 66,000 participants with diabetes, does not also confirm a greater risk for lung cancer in patients with diabetes than in the general population [19].

Our lost to follow-up patients, till the time they were last examined, had very similar characteristics to our regularly followed-up patients. Their cancer comorbidity was also very similar, strengthening the value of our observation, since it refers to 1,709 patients with T2DM and 236 patients with T1DM of at least 10-year duration, followed up at a single center.

Whereas cardiovascular complications are well known for diabetes and all our efforts focus on their prevention, the frequent coexistence of diabetes and cancer does not seem to have been consolidatedin the thought of doctors treating subjects with diabetes. On the other hand, subjects with diabetes undergo yearly full clinical and laboratory examination for the detection of the possible emergence of "classical" complications. Because of the amplitude of this control, many patients derive, unfortunately, the wrong impression that they have undergone all presymptomatic control necessary for their health [20]. The question is whether frequent coexistence of diabetes with malignancies should be considered as a "new," unappreciated till now, diabetes complication. In that case, doctors dealing with diabetes will strengthen their efforts to educate their patients in the strategies of cancer prevention, the way they do for cardiovascular diseases' prevention strategies. It is noteworthy that these strategies have a lot in common (prevention

Table 3: Data of all patients with at least 10-year diabetes duration examined for the first time during the last 25 years but lost to follow-up after at least one-year regular visits.

N	T2DM		T1DM	
	950 (All)	CA + (n = 101) 10.6%	102 (all)	CA + (n = 2) 2.0%
Sex (male)	545 (57.4%)	54 (54.5%)	61 (59.8%)	
Age	67.9 ± 0.3	69.7 ± 0.9	41.6 ± 1.4	57.5 ± 9.5
Age of diabetes onset	50.2 ± 0.3	53.0 ± 1.1	21.7 ± 1.4	25.5 ± 7.5
Diabetes duration	18.0 ± 0.2	19.3 ± 0.9	19.9 ± 0.9	32.0 ± 2.0
OADs (%)	652 (68.6%)	63.4%		
OADs + basal insulin	22 (2.3%)	1.0%		
Insulin treated	276 (29.1%)	35.6%		
Years of DM before insulin initiation	15.1 ± 0.4	13.9 ± 1.2		
A1c	7.5 ± 0.0	7.2 ± 0.1	8.4 ± 0.2	6.5 ± 1.2
BMI	27.9 ± 0.1	28.0 ± 0.5	25.5 ± 0.4	22.6 ± 0.0

OADs: oral antidiabetic drugs.

Table 4: Types of cancer in T2DM patients lost to follow-up.

Types of cancer (n = 101)	Frequency order of the cancer type in the regularly followed-up T2DM population	%
Prostate cancer	1	14.9%
Breast cancer	2	12.9%
Urinary tract	3	12.9%
Colon cancer	5	11.9%
Hematological malignancies	4	7.0%
Pancreatic cancer	10	6.9%
Female genital cancer	7	6.0%
Skin cancer	9	5.0%
Lung cancer	6	3.0%
Thyroid cancer	8	3.0%
Rest of GI; rest of URT; unknown		16.5%

of obesity, increase of physical activity, choice or rejection of certain foodstuff, etc.). Also they will probably include in the annual patients' evaluation the acceptable examinations for presymptomatic cancer detection, depending on each patient's sex, age, and familial anamnesis.

In conclusion, patients with T2DM of at least 10-year duration regularly followed up at our clinic exhibit high-cancer comorbidity, while patients with T1DM do not. Importantly, no specific characteristics can discriminate patients with cancer; therefore, presymptomatic cancer detection and prevention strategies may have to be reinforced and incorporated into the annual systematic evaluation of subjects with diabetes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Rectal Sensitivity in Diabetes Patients with Symptoms of Gastroparesis

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In a clinical setting, diabetic autonomic complications (cardiac, gastrointestinal, urogenital, etc.) are often handled as separate entities. We investigated rectal sensitivity to heat, mechanical distension, and electrical stimulations in 20 patients with diabetes and symptoms of gastroparesis, to evaluate the extent of visceral neuronal damage. Furthermore, to evaluate the relation between the nervous structures we examined gastric emptying and cardiac autonomic function with the hypothesis being an association between these. We found that 60% of patients had delayed gastric empting. Rectal hyposensitivity was a general finding as they tolerated 67% higher thermal, 42% more mechanical, and 33% higher electrical current intensity compared to healthy controls. In patients, most heart rate variability parameters were reduced; they reported significantly more gastrointestinal symptoms and a reduced quality of life in all SF-36 domains. Shortened RR interval correlated with reduced rectal temperature sensitivity, and gastric retention rate was negatively associated with symptoms of nausea and vomiting. To conclude, in these patients with signs and symptoms of diabetic gastroparesis, rectal sensitivity was reduced, and heart rate variability was impaired. Thus, we suggest regarding diabetic autonomic neuropathy as a diffuse disorder. Symptoms of widespread autonomic dysfunction and sensory disorders should be expected and treated in these patients.

1. Introduction

Gastrointestinal (GI) complaints are more common in all types of diabetes mellitus (DM) patients compared to the general population [1, 2]. Symptoms such as pain, bloating, excessive fullness, vomiting and diarrhea may range from mild and intermittent to severe and life-threatening. Treatment options are limited (diet, pharmacological, and invasive procedures) and frequently incapable of adequate symptom relief [3]. Recent years have seen an improved understanding of the multiple coexisting pathophysiological mechanisms behind the symptoms. In addition to peripheral autonomic neuropathy and affection of the sensory visceral nerves, functional changes have also been detected in the brain networks encoding visceral pain [4, 5]. Mirroring this, magnetic resonance imaging techniques have revealed altered brain microstructure in the so-called "pain matrix" of the brain [6, 7]. Other mechanisms include altered elasticity of the GI wall, enterohormonal changes, anxiety/depression, exocrine pancreatic insufficiency, bacterial imbalance, autoimmunity,

loss of interstitial cells of Cajal, and the direct effects of hyperglycemia on GI motility [8–10]. In line with this complex pathophysiology, the association between upper GI symptoms and gastric emptying is modest. Conversely, gastric emptying rate cannot explain the range of upper GI symptoms experienced, neither in DM patients nor in the case of idiopathic gastroparesis [11–14].

Until now, the majority of studies in this field have focused on diabetes complications of the upper GI tract. Knowledge about the extent of damage to the lower GI tract is sparse; however, in one of our recent studies we demonstrated rectal hyposensitivity in patients suffering from diabetic *sensorimotor* neuropathies [15]. A limited number of studies have examined the rectal sensitivity to distention in diabetes patients with fecal incontinence; however, none have employed multimodal sensory investigations with the possibility to investigate several nerve fibres and pathways [16, 17].

We hypothesised that DM patients with upper GI symptoms are hyposensitive in the distal GI tract and that visceral sensitivity, gastric emptying rate, cardiac autonomic function, and clinical symptoms would be associated. Thus, the main aim of this study was to examine the rectosigmoid sensitivity to multiple modalities (heat, mechanical distension, and electrical stimulations) in diabetes patients with symptoms of upper GI dysmotility. Furthermore, we aimed to characterise these patients in terms of cardiac autonomic parameters, gastric emptying rate, quality of life, and GI symptom scores.

2. Research Design and Methods

2.1. Subjects. Twenty diabetes patients were included between August 2010 and October 2011 from the outpatient clinic at Haukeland University Hospital. Inclusion criteria were upper GI symptoms refractory to treatment, type 1 or type 2 DM, and age between 18 and 65 years. All patients had previously undergone a gastroscopy in order to rule out other causes of their complaints. Major exclusion criteria were implanted gastric electrical stimulation device, nonneuropathic pain conditions, uremia, alcohol abuse, and unwillingness to cease analgesics or prokinetics prior to sensory examinations. Two patients were unable to tolerate the rectosigmoid probe, but completed the other parts of the study. As a control group, 16 healthy volunteers without GI complaints were recruited from the medical departments at Bergen and Aalborg University Hospitals. Clinical characteristics of the study population are summarized in Table 1. Oral and written consent was obtained from all participants, and the study was approved by the local ethics committees (Regional Etisk Komité Vest 2010/2562-6 and Aalborg N-20090008).

2.2. Gastric Emptying. Prior to the experimental rectal sensory assessments, all patients had their gastric emptying rates evaluated. Twenty spherical radiopaque markers (diameter 4 mm, density 1.27 g/mm^3) where given together with a standardized breakfast. The number of markers still present in the stomach was determined by the help of fluoroscopy after 4, 5, and 6 hours, enabling the calculation of an average retention rate. Gastroparesis was defined as a retention rate >26% in males and >63% in females through the 4–6 hour period. The method has a sensitivity of 34% and specificity of 97% compared to scintigraphy and has been further described and validated elsewhere [11].

Table 1: Clinical characteristics.

Variables	Patients (n = 20)	Controls (n = 16)
Age (years)	44.5 (±9.6)	44.8 (±9.3)
Gender (male/female)	5/15	5/11
Body mass index (kg/m^2)	26.5 (±5.1)	24.4 (±3.4)
Diabetes duration (years)	26.5 (±9.9)	—
Diabetes type (1/2)	17/3	—
HbA1c (%)	9.7 (±2.1)	5.6 (±0.2)
Smoking status (never/past/present)	10/4/5	10/6/0
Retinopathy (%)	65	—
Known neuropathy (%)	55	—
Known cardiovascular disease (%)	20	0
Creatinine level (IQ-range) (μmol/L)	69.0 (58.0–104.0)	72.0 (66.5–78.0)
Beta-blocker (%)	20	0
ACEI/angiotensin receptor blocker (%)	45	6
Statin use (%)	65	6

Data are means (±SD) unless otherwise indicated.
ACEI = angiotensin converting enzyme inhibitor.

2.3. Rectal Sensory Assessment. On a separate study day, participants were instructed to fast for at least 6 hours prior to sensory examinations. In order to avoid the effect of glucose and insulin levels on GI sensations, both DM patients and healthy controls underwent a euglycemic hyperinsulinemic clamp procedure [18]. Sensory assessments were performed upon achieving the target blood glucose of 6 mM. Participants were instructed to grade the sensations using a modified Visual Analogue Scale (VAS). The scale is well known, validated, and has been employed in several studies on both upper and lower GI sensations. It runs from zero to ten, with 1 being the detection threshold, 3 being a definite moderate sensation, 5 the pain threshold, 7 moderate pain, and 10 unbearable pain [19]. After clearing the rectum with a suppository (Klyx, Ferring AS, Copenhagen, Denmark), a multimodal rectal probe (Ditens A/S, Aalborg, Denmark) was inserted through a small anoscope [20]. The probe, measuring 6.2 mm in diameter, had two channels for circulating or filling water into a noncompliant 30 μm thick polyester urethane balloon placed near the tip of the probe. Two separate channels contained a thermometer and a pressure sensor. Furthermore there were two electrodes placed at the tip for electrical stimulations. Details of the probe design have been described previously [20]. Rectal thermal sensitivity was investigated by circulating 68°C water inside the rectal bag prefilled with 60 mL of 37°C water, thus enabling a gradually rising temperature. Accurate circulation flow rate (150 mL/minute) was ensured by a peristaltic

pump (Ole Dich Instrument Makers, Hvidovre, Denmark). The temperature and time of circulation needed to reach sensations corresponding to 1, 3, 5, and 7 on the VAS were recorded. At VAS = 7 the heated water was immediately evacuated to minimize participant discomfort. Mechanical sensitivity was tested after emptying the rectal bag completely then infusing 37°C water at a constant rate of 200 mL/minute into the rectal bag, using the same peristaltic pump as for thermal stimulations. Three preconditioning stimulations to pain detection threshold were performed to minimise the effect of viscoelastic properties of the rectum to the mechanical distensions [20]. During the fourth stimulation the actual sensory assessment was performed, and time of filling and bag pressure needed to reach 1, 3, 5, and 7 on the VAS were recorded. When patients reached the sensation corresponding to 7 on the VAS, the pump was reversed and the bag was emptied at the same rate. Time of emptying from VAS 7 to VAS 0 was also recorded. Although the study was not principally designed to examine the rectal biomechanical properties, the rectal compliance was estimated according to a method previously described [21]. Electrical sensitivity assessment commenced after ensuring adequate mucous membrane contact by measuring the interelectrode impedance (ideally $\leq 2\,k\Omega$). In case of higher impedance, the probe was gently manipulated and the electrical contact reassessed. The electrical stimuli were administered as 2 ms square pulses via a computer-controlled constant current stimulator (DIGITIMER Ltd., Welwyn Garden City, UK), starting at subdetection levels and increasing in increments of 1 mA. Intermittently, sham stimuli or a lower current intensity was administered, in order to limit the effect of anticipation and expectation. Participants were asked to report when the rectal sensation reached 1, 3, 5, and 7 on the VAS, and the corresponding current intensities were recorded. This multimodal sensory assessment of the rectum has been validated and described in greater detail elsewhere [20].

2.4. Heart Rate Variability. For evaluation of the heart rate variability—a measure of the cardiac autonomic nervous system—a 24 hour Holter ECG recording was performed in all participants (Schiller MT-200, Schiller AG, Baar, Switzerland). The following time-domain parameters where calculated: (1) RR intervals (representing the average heart rate), (2) standard deviation of normalized RR intervals (SDNN—representing the total variability), (3) standard deviation of 5-minute segments of normalized RR intervals (SDANN), (4) root mean square of the differences between successive normalized RR intervals (RMSSD—primarily representing the parasympathetic activity), and (5) the percentage of normalized RR intervals that differ more than 50% compared to the previous ones (pNN50—representing the parasympathetic dominance over the sympathetic activity) [22].

2.5. Questionnaires. All participants completed two questionnaires. To evaluate GI symptoms, we used the Patient Assessment of Upper Gastrointestinal Disorder Severity Symptom Index (PAGI-SYM). It consists of 20 questions, and symptoms in the preceding two weeks are graded from 0 (no symptoms) to 5 (very severe symptoms). In addition to a total score, six subscales were calculated: postprandial fullness/early satiety, nausea/vomiting, bloating, upper abdominal pain, lower abdominal pain, and heartburn/regurgitation [23]. Furthermore, the Short Form-36 (SF-36) was employed to investigate health-related quality of life. Both questionnaires have been previously translated, validated, and extensively used in Norwegian.

2.6. Statistics. Statistical analyses were performed in SigmaPlot 11 (Systat Software Inc., San Jose, CA, USA), using a P value of ≤ 0.05 as significance level. Results are given as means ± standard error of mean or if not normally distributed as median (interquartile (IQ) range) unless otherwise specified. To compare overall rectal sensitivities, a two-way analysis of variance (ANOVA) was employed with the factors pain modality and VAS level. When comparing the patients and healthy controls in terms of baseline characteristics, gastric emptying rate, heart rate variability, and questionnaires, one-way ANOVAs were performed. Data that were not normally distributed were compared by Kruskal-Wallis' method. Correlations between rectal sensitivity, gastric emptying, GI symptoms, and heart rate parameters were investigated by Spearman's rank order test.

3. Results

3.1. Gastric Emptying Rates. Radiopaque marker (ROM) examination was performed in all patients and was positive for gastroparesis in 60% (12 out of 20). The mean 4–6 hour ROM retention rate in women (56.4 ± 9.5%) was numerically higher than in men (40.4 ± 15.5%); however, this was not statistically significant ($P = 0.41$).

3.2. Rectosigmoid Sensitivity and Compliance. All participants were successfully clamped, and the mean blood glucose levels were similar during testing (patients 6.4 ± 0.12 and controls 6.1 ± 0.13 mmol/L, $P = 0.12$). Rectal sensitivities are summarized in Figures 1(a)–1(c). In short, diabetes patients needed significantly higher temperatures to induce the various VAS-levels compared to controls; all VAS-levels average temperature was 49.2 ± 0.80°C in patients and 45.0 ± 0.88°C in healthy controls ($F = 12.8$, $P < 0.001$). Similarly, the duration of thermal stimulation was longer in patients than in controls (74.4 ± 5.3 seconds versus 44.6 ± 5.7, $F = 14.8$, $P < 0.001$). For mechanical sensitivity there were similar results; at all VAS-levels average rectal balloon volume was 215 ± 13 mL in patients and 151 ± 13 mL in healthy controls ($F = 11.9$, $P < 0.001$). This corresponded to higher mean rectal balloon pressure at the various VAS levels in the patient cohort (22.3 ± 1.7 cm H_2O) than in the control cohort (15.4 ± 1.7 cm H_2O, $F = 8.0$, $P = 0.005$). However, there was no difference in compliance between patients (median 0.024 (0.020–0.048) mL/cm H_2O) and controls (median 0.029 (0.023–0.062) mL/cm H_2O), $P = 0.38$.

The diabetes patients were also hyposensitive to electrical stimulation and needed significantly higher current intensities to reach the predefined VAS levels. All VAS levels

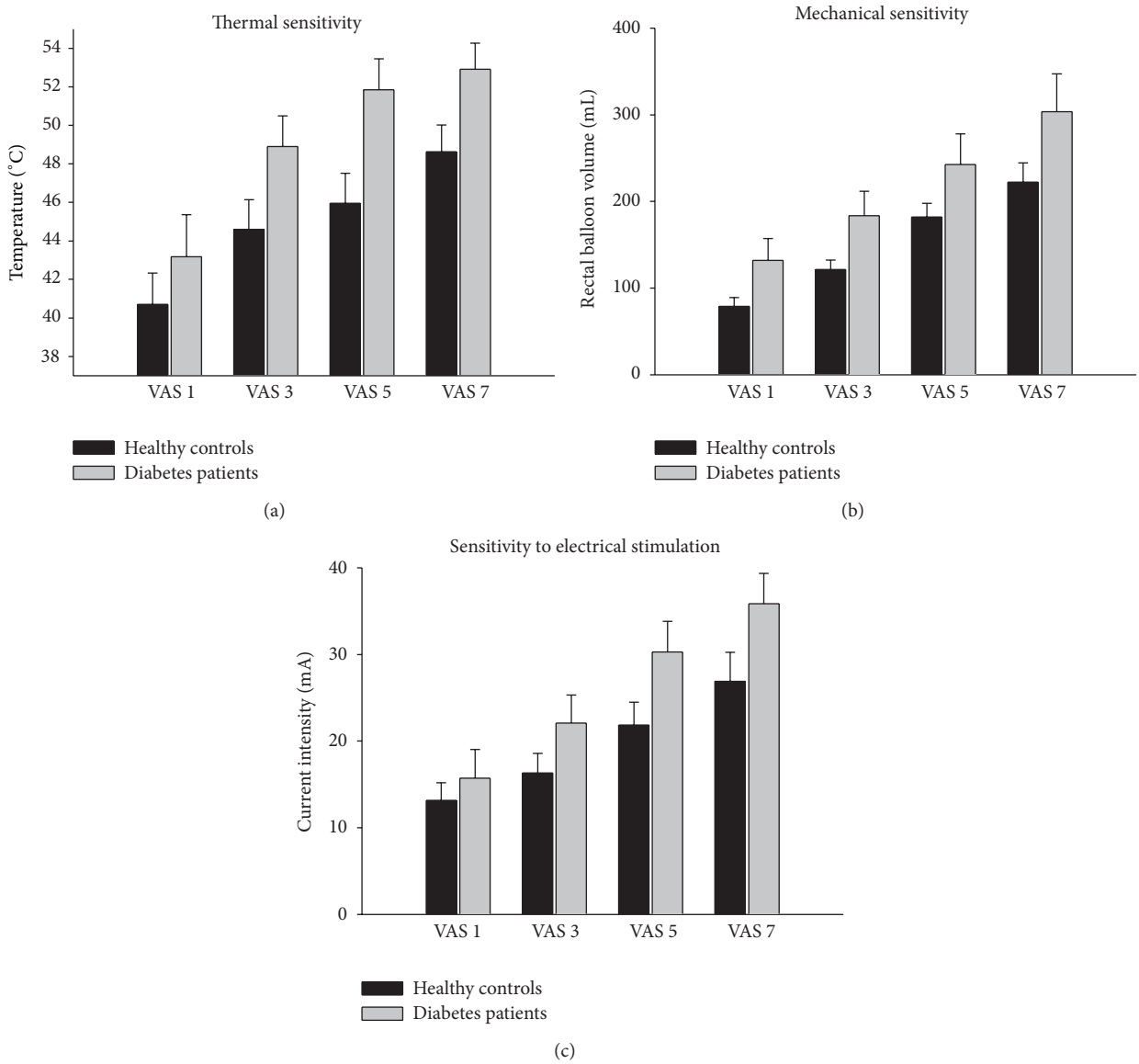

Figure 1: (a) The rectal sensitivity to thermal stimulation. Patients showed overall hyposensitivity to heat ($F = 12.8$, $P < 0.001$). The Y-axis describes the actual balloon temperature needed to induce the sensation corresponding to VAS ratings. Error bars represent SEM. (b) The rectal sensitivity to mechanical stimulation. Patients showed overall hyposensitivity to mechanical distension ($F = 11.9$, $P < 0.001$). The Y-axis describes the rectal balloon volumes needed to induce the corresponding VAS ratings. Error bars represent SEM. (c) The rectal sensitivity to electrical stimulation. Patients showed overall hyposensitivity to electrical stimulation ($F = 8.8$, $P < 0.004$). The Y-axis describes the current intensity needed to induce the corresponding VAS scores. Error bars represent SEM.

average current intensity was 26.0 ± 1.5 mA in patients and 19.6 ± 1.7 mA in healthy controls ($F = 8.8$, $P < 0.004$). In relative terms, diabetes patients needed 67% and 42% more time of thermal and mechanical stimulations, and 33% higher electrical current intensities to reach the same VAS levels as the healthy controls.

3.3. Heart Rate Variability. Technically acceptable 24-hour Holter results were obtained in 18 patients and 11 healthy volunteers. The heart rate was higher in patients (mean RR interval in patients 745 ± 106 ms compared to 820 ± 79 ms in healthy controls, $P = 0.05$). Also, most parameters of heart rate variability were reduced in patients: median SDNN 102 ms (interquartile range 71–118) versus 137 ms (118–168), $P = 0.02$; median SDANN 83 ms (60–96) versus 128 ms (98–141) $P = 0.004$. Median pNN50 was reduced in patients 2.5% (1.1–6.0) versus 13.0% (3.0–24.4) in healthy controls, $P = 0.02$. There was no difference between the two groups in terms of RMSSD, in patients 28 ms (17–46) and in controls 38 ms (23–52), $P = 0.29$.

3.4. Questionnaires. The patients scored significantly higher in all of the investigated aspects of upper and lower gastrointestinal symptoms (all $P < 0.001$); see Table 2. The SF-36 questionnaire revealed a strongly reduced self-reported

Table 2: PAGI-SYM scores.

		Patients	Healthy controls
Subscale item	Postprandial fullness	3.50 (2.75–4.0)	0.25 (0.0–0.44)
	Nausea/vomiting	1.33 (0.50–3.25)	0.0 (0.0-0.0)
	Bloating	3.50 (3.50–4.75)	0.0 (0.0-0.0)
	Upper abd. pain	2.76 (±0.40)	0.10 (±0.07)
	Lower abd. pain	2.00 (1.00–3.50)	0.0 (0.0-0.0)
	Heartburn/regurg.	1.14 (0.86–2.61)	0.0 (0.0-0.0)
Total score		2.25 (1.54–2.91)	0.05 (0.0–0.23)

Results of the patient assessment of upper gastrointestinal disorder severity symptom index (PAGI-SYM) questionnaire. Twenty patients and 15 healthy controls completed the questionnaire. Abd. = abdominal, regurg. = regurgitation. All $P < 0.001$.

Table 3: SF-36 scores.

		Patients	Healthy controls
Subscale item	Physical functioning	72.5 (40.0–85.0)	100.0 (100.0-100.0)
	Role lim. phys. (RP)	0.0 (0.0–50.0)	100.0 (100.0-100.0)
	Bodily pain	41.6 (±26.6)	88.5 (±12.6)
	General health	33.4 (±19.8)	85.2 (±16.6)
	Energy fatigue/vitality	32.5 (±18.4)	75.7 (±13.7)
	Social functioning	62.5 (37.5–75.0)	100.0 (100.0-100.0)
	Role lim. emot. (RE)	100.0 (33.3–100.0)	100.0 (100.0-100.0)
	Mental health (MH)	76.0 (68.0–80.0)	84.0 (76.0–92.0)
Summary scores	Physical comp. (PCS)	33.3 (18.8–39.2)	55.8 (54.0–57.3)
	Mental com. (MCS)	47.7 (42.9–50.0)	53.1 (48.5–55.3)

Results of the Short Form-36 questionnaire, presented as median (IQ-range) or mean (±SD). Eighteen patients and 15 healthy controls completed the questionnaire. RP = role limitations due to physical health, RE = role limitations due to emotional problems, PCS = physical component summary and MCS = mental component summary.
All $P < 0.001$ except RE: $P = 0.02$, MH: $P = 0.04$, and MCS: $P = 0.02$.

health in the patient cohort with all SF-36 subscales reduced (all $P < 0.05$). For details, please see Table 3.

3.5. Clinical Correlations. According to our hypothesis, we investigated the associations between rectal sensitivity, gastric emptying, heart rate parameters, and gastrointestinal symptoms. There were no statistically significant correlations between rectosigmoid sensitivity and gastrointestinal symptoms (PAGI-SYM). The gastric retention rate was positively associated with the temperature sensitivity ($r = 0.59$, $P = 0.02$); that is, the more delayed the gastric emptying, the more increased the rectal sensitivity to temperature. There was a similar trend in mechanical pressure, but it did not reach statistical significance ($r = 0.44$, $P = 0.08$). Gastric retention was also negatively associated with symptoms of nausea and vomiting ($r = -0.51$, $P = 0.03$); that is, the more delayed the gastric emptying, the *less* the symptoms. The RR interval was negatively associated with symptoms of postprandial fullness ($r = -0.49$, $P = 0.04$) as well as the rectal temperature sensitivity ($r = -0.63$, $P = 0.01$); that is, patients with higher mean heart rate had more symptoms of fullness and reduced rectal sensitivity to heat. No other associations could be detected between these predefined parameters.

4. Discussion

We have shown that patients with symptoms and signs of diabetic gastroparesis had sensory deficits in the distal gastrointestinal tract, indicating a widespread nature of visceral neuropathy. Furthermore, patients had reduced heart rate variability and increased mean heart rate, a sign of extensive autonomic dysfunction in DM. Differences in rectosigmoid compliance between DM patients and controls were not detected.

Major limitations include the relatively low number of study subjects and the mixed type 1 and type 2 DM cohort. Although the two conditions share some specific pathogenetic traits (in particular hyperglycemia), the symptom presentation and gastric emptying rate may differ slightly. Type 1 DM patients have been shown to be more prone to vomiting, whereas type 2 patients have relatively more nausea. On average, gastric retention is more pronounced in type 1 DM, although the differences are subtle [24]. Method specific limitations are discussed below.

Unlike the colon, the rectum receives innervation from both visceral (sacral) and somatic (pudendal) nerves. In this study we wanted to investigate the visceral afferents specifically, and the probe was positioned at least 15 cm above the anus, thus limiting involvement of the lower somatic nerve afferents. Although rectal sensations by nature primarily deal with the feeling of fullness and the urge to defecate, we chose a multimodal approach in order to obtain a comprehensive sensory profile. Thermal stimulation has the advantage of being highly reproducible. It stimulates the mucosal receptors directly, although it is probably less physiological in nature. Mechanical stimulation, on the other hand, is more physiological but also depends on the varying elastic properties of the rectum, the muscular tone, and neuromuscular feedback loops. Finally, electrical stimulation is highly reproducible; it bypasses the peripheral receptors entirely and depolarizes the nerve endings directly [20, 25].

Gut sensitivity and motility are subject to modification by glucose and insulin levels. Both act as sensitizers to stretch in the stomach [26]. Our previous studies indicate a role for insulin, but not glucose, in the sensitivity to electrical stimulation in the esophagus [18, 27]. The anorectal region is less well investigated in this respect, and results are somewhat contradictory (increased, decreased, or no effect of hyperglycemia on sensitivity) [28–30]. To avoid any interference, we decided to use a hyperinsulinemic euglycemic clamp technique in all study subjects. This setup combined

with multimodal rectal sensory assessment has been used in several previous studies and yields reproducible and physiologically meaningful results [4, 15]. In this study, no difference between diabetes patients and controls could be detected in terms of the rectosigmoid compliance; that is, the mechanosensory findings were likely unrelated to changes in rectal wall properties. Some previous studies have found reduced rectal compliance in DM, but this seems to have been strongly influenced by the actual blood glucose levels [16, 28]. In our study, euglycemia was ensured by a hyperinsulinemic clamp technique, possibly influencing our findings.

Reduced sensitivity in diabetes patients is not necessarily the product of *peripheral* nerve pathology. Altered structure and/or activity at central nervous system levels are undoubtedly present in patients with both somatic and autonomic neuropathies, as has been demonstrated by several MRI studies [7, 31–33]. Furthermore, electrophysiological studies using evoked potentials with advanced brain activity modeling have also detected abnormal brain activity patterns in patients with diabetes, autonomic neuropathy, and gastrointestinal symptoms [4, 34]. Indeed, a subset of subjects included in this study has previously been investigated using electrically induced evoked brain potentials, where functional brain changes could be detected [5]. Still, at least some degree of peripheral pathology can be argued for, as the degree of hyposensitivity varies according to the mode of stimulation; that is, the least hyposensitivity was detected in the case of electrical stimulation—bypassing the mucosal receptors, possibly indicating a noncentral involvement.

This study demonstrated that diabetes patients with symptoms of *upper* GI dysfunction have a widespread visceral hyposensitivity. Thus, it is plausible—although not yet proven—that DM affects nerves in the entire GI tract. Although clearly multifactorial, diabetic autonomic neuropathy—in particular affecting the vagal nerve—has been implicated in *upper* GI dysfunction. The afferent visceral sensory nerves innervating the *lower* GI tract travel a relatively short distance through the pelvic splanchnic nerves to the sacral parts of the spinal cord. Thus, these two visceral nerve pathways are distinct in terms of localization, length, and distribution. Our results suggest that nervous dysfunction in DM is not limited to the well-investigated vagal nerve but may also include the shorter splanchnic nerves. This implicates that small fiber diabetic neuropathy possibly is less length-dependent than large-fiber sensorimotor polyneuropathies. This, in turn, might help explain the varied clinical presentations of visceral complications in DM [35]. From a clinicians' perspective, diverse neuropathic complications should be actively considered, once a patient presents symptoms of autonomic dysfunction in any organ system.

In this explorative study, we only investigated the associations in line with our hypothesis. Gastric retention showed *positive* association with rectal sensitivity and *negative* association with the feeling of gastric fullness. That is, the more delayed the gastric emptying, the less the symptoms and the higher the rectal sensitivity to heat. Although somewhat surprising, our results are in line with a number of studies which have found very weak—if any—correlation between gastric emptying rates and symptoms of diabetic gastroparesis [36, 37]. Indeed, there is an ongoing controversy surrounding the relationship between these parameters, which strongly affects clinical intervention trials [38, 39]. Finally, the method of investigating gastric emptying—by radiopaque markers—may be less sensitive and show moderate correlation to the gold standard, scintigraphy, although the latter has shown similar poor association to upper gastrointestinal symptoms [11, 40]. Among heart rate parameters, the RR interval was associated with visceral sensitivity, that is, reduced thermal sensitivity with increasing heart rate. Increased mean heart rate is a well-known early marker of cardiac autonomic neuropathy, so any association with visceral sensitivity is in line with a pathophysiological explanation involving autonomic neuropathy [35]. A limitation of the present study in this respect is the lack of spectral frequency analysis—a method which might detect more subtle cardiac autonomic imbalance [41].

Diabetic gastrointestinal complications are challenging to investigate and diagnose. This is partly not only due to the inaccessibility of GI organs but also due to the imprecise nature of GI motility measurements and the poor correlation between symptoms, GI dysmotility, and autonomic neuropathy [42, 43]. Also, GI symptoms are so common in general that the clinical question of diabetic GI complications frequently arises. The invasive sensory investigations performed by our group in this and previous studies have demonstrated a concomitant affection of upper and lower GI tract, in conjunction with cardiac autonomic dysfunction [5]. Investigating the heart rate variability through electrocardiography or other parameters of cardiac autonomic function is easy and hazard-free for the patient. Due to multiple weak associations between various autonomic dysfunctions, these investigations are not likely to offer meaningful positive predictive information as far as diabetic gut dysfunctions are concerned. On the other hand, *normal* cardiac autonomic function might indicate that the GI signs and symptoms are *not* caused by autonomic dysfunction. This would make heart-rate variability testing a logical first step to screen diabetic from nondiabetic GI complaints. Further studies are warranted to test this hypothesis.

In conclusion, this study provided evidence of the generalized nature of diabetic autonomic neuropathy. Diabetes patients with signs and symptoms of upper GI dysfunction displayed reduced rectal sensitivity to heat and mechanical and electrical stimulation. Also, the heart rate variability was impaired. In a clinical setting, the presence of autonomic dysfunction could be regarded as a diffuse neuropathic complication.

Abbreviations

ANOVA: Analysis of variance
BMI: Body mass index
DM: Diabetes mellitus
ECG: Electrocardiography
GI: Gastrointestinal
IQ-range: Interquartile range
MRI: Magnetic resonance imaging

PAGI-SYM: Patient assessment of upper gastrointestinal disorder severity symptom index
ROM: Radiopaque marker
SEM: Standard error of the mean
SD: Standard deviation
SF-36: Short Form-36
VAS: Visual analogue scale.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Christina Brock, Magnus Simrén, Jens B. Frøkjær, and Asbjørn M. Drewes participated in study design; Eirik Søfteland, Christina Brock, Jens B. Frøkjær, and Georg Dimcevski participated in data collection; Eirik Søfteland, Christina Brock, and Georg Dimcevski participated in data analysis; all coauthors participated in interpretation of results; Eirik Søfteland and Georg Dimcevski participated in paper preparation. All coauthors participated in critical revision of paper. Eirik Søfteland is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Research Article

The Effect of Diabetes Self-Management Education on Body Weight, Glycemic Control, and Other Metabolic Markers in Patients with Type 2 Diabetes Mellitus

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Aims. To comprehensively evaluate the effect of a short-term diabetes self-management education (DSME) on metabolic markers and atherosclerotic parameters in patients with type 2 diabetes. *Methods.* 76 patients with type 2 diabetes were recruited in this study. They were divided into the intervention group ($n = 36$) and control group ($n = 40$). The patients in the intervention group received a 3-month intervention, including an 8-week education on self-management of diabetes mellitus and subsequent 4 weeks of practice of the self-management guidelines. The patients in the control group received standard advice on medical nutrition therapy. Metabolic markers, carotid intima-media thickness (CIMT), and carotid arterial stiffness (CAS) of the patients in both groups were assessed before and after the 3-month intervention. *Results.* There was a significant reduction in hemoglobin A1c (HbA1c, $-0.2 \pm 0.56\%$ versus $0.08 \pm 0.741\%$; $P < 0.05$) and body weight (-1.19 ± 1.39 kg versus -0.61 ± 2.04 kg; $P < 0.05$) in the intervention group as compared to the control group. However, no significant improvements were found in other metabolic markers, CIMT and CAS ($P > 0.05$). *Conclusions.* DSME can improve HbA1c and body weight in patients with type 2 diabetes.

1. Introduction

Diabetes mellitus, commonly type 2 diabetes mellitus, is an increasing health problem worldwide. It has been estimated that there will be 552 million patients with diabetes and 300 million people with impaired glucose tolerance in 2030 [1]. Diabetes mellitus is associated with various atherosclerotic complications, including cerebrovascular and cardiovascular diseases, causing significant morbidity and mortality.

Monitoring of metabolic markers, such as blood pressure, body weight, lipid profile, blood glucose, and HbA1c, is essential in the clinical management of patients with diabetes, because hypertension, obesity, and dyslipidemia are well-known risk factors of atherosclerosis and are common in diabetic patients [2]. Monitoring of these risk factors also helps in the evaluation of treatment response of the patients. In addition, carotid intima-media thickness (CIMT) and carotid arterial stiffness (CAS) are atherosclerotic parameters which are usually considered as the predictors of cardiovascular and cerebrovascular events [3, 4]. Patients with diabetes tend to have increased CIMT and CAS [5, 6]. Therefore, assessing these atherosclerotic parameters is important for patients with diabetes to evaluate the risk of cardiovascular and cerebrovascular events as well as treatment response.

As a long-term disease, diabetes mellitus needs lifetime care and management. However, 50–80% of patients with diabetes did not have enough skills and knowledge for self-care of the disease [7]. Therefore, diabetes self-management education (DSME) plays an important role in the clinical management of diabetes. Previous studies have shown that DSME improves homeostasis of metabolism of the patients, and healthy lifestyles prevent the development of atherosclerosis in patients with type 2 diabetes [8, 9]. However, there is limited information in the literature about the effects of DSME on both metabolic markers and atherosclerotic parameters. Therefore, this study was undertaken to comprehensively

Table 1: The content of the diabetes self-management education for the patients in the intervention group.

	Contents
Healthy eating	(i) Common misunderstanding of diet for self-management of type 2 diabetes mellitus
	(ii) The types of healthy and unhealthy foods for type 2 diabetics
	(iii) The benefits of health foods and the drawbacks of unhealthy foods for type 2 diabetics
	(iv) Recognition of healthy foods based on food package
	(v) Healthy cooking methods for type 2 diabetics
	(vi) Appropriate caloric intake for type 2 diabetics
	(vii) A brief method for counting calories of foods
	(viii) The best time to eat
Being active	(i) The importance of regular exercise for type 2 diabetics
	(ii) Common misunderstanding of exercise for self-management of type 2 diabetes mellitus
	(iii) Suitable types of exercise for type 2 diabetics
	(iv) Individualized plan for regular exercise
	(v) Self-check and control of body weight
Monitoring	(i) The importance of regular monitoring of blood glucose
	(ii) Methods of self-monitoring of blood glucose
	(iii) Self-management of blood glucose
Taking medication	(i) Pathology and medical treatments of type 2 diabetes mellitus
	(ii) The importance of taking diabetic medications
	(iii) Efficacies and side effects of different diabetic medications
	(iv) The appropriate time and frequency of taking diabetic medications
Problem solving	(i) Choosing healthy foods under various circumstances
	(ii) Doing appropriate exercises according to individual health status
	(iii) Methods to handle abnormal blood glucose
Reducing risks	(i) Common complications of type 2 diabetes mellitus
	(ii) Risk factors of diabetic complications
	(iii) The importance of stopping unhealthy behaviors (e.g., smoking) and maintaining healthy lifestyles
Healthy coping	(i) The importance of self-management of type 2 diabetes mellitus
	(ii) Designing an individualized plan for self-management of type 2 diabetes mellitus

investigate the effect of DSME on metabolic markers and atherosclerotic parameters in patients with type 2 diabetes.

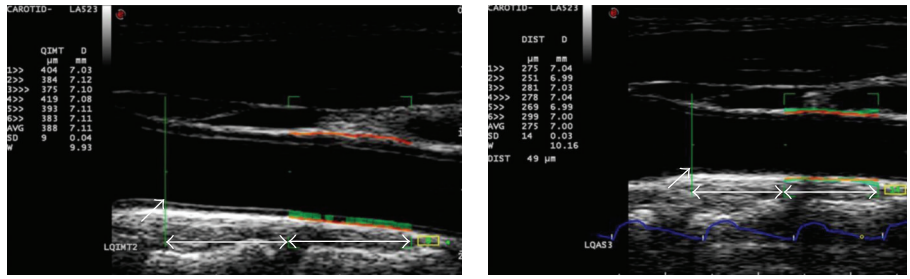
2. Methods

2.1. Subjects. In the present study, a total of 88 patients with type 2 diabetes were recruited from a local Chinese non-profit-making organisation for diabetics (Angel of Diabetic, Hong Kong). The patients were randomly assigned into the intervention group ($n = 44$) or the control group ($n = 44$). Blocked randomization was used in the present study, and allocation sequence was concealed from researchers and patients. The inclusion criteria included Chinese and adult (>18 years old) having type 2 diabetes for more than one year. Criteria for exclusion were attendance of previous diabetes self-care courses, radiotherapy of the neck, carotid endarterectomy, and carotid stenting. Among the 88 patients, 12 of them (8 in the intervention group and 4 in the control group) did not complete the DSME programme or did not attend the follow-up examination and thus were excluded from the study. Therefore, finally 76 patients with type 2 diabetes were included in the study (intervention group, $n = 36$; control group, $n = 40$).

This study was approved by the Human Subject Ethics Subcommittee of The Hong Kong Polytechnic University. Patients were informed with the details of the study and written consent was obtained from the patients before they participated in the study.

2.2. Intervention. The patients in the intervention group participated in a DSME programme in which the patients needed to attend a 2-hour lesson weekly for eight weeks and to follow the self-management guidelines of the education programme in the daily activities within the study period. The patients in the control group did not attend any lessons of the DSME. However, they still received standard advice on medical nutrition therapy. For the DSME programme, all the lessons were conducted by a certificated nutritionist and were focused on the skills and knowledge for healthy eating, being active, monitoring, taking medication, problem solving, reducing risks, and healthy coping (Table 1) [10].

2.3. Blood Test. Blood test was conducted twice for each patient. For each blood test, fasting venous blood sample was obtained from the patient by venipuncture to evaluate the whole blood level of metabolic markers: total triglyceride,



(a) (b)

Figure 1: The assessment of intima-media thickness and arterial stiffness in the carotid artery. (a) Radiofrequency-based quality intima-media thickness. (b) Radiofrequency-based quality arterial stiffness. The arrows indicate the inferior end of the carotid bulb and the double-arrow lines show a distance of 1 cm.

total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), blood glucose, and hemoglobin A1c (HbA1c). All the metabolic marker analyses were conducted using an automated clinical chemistry analyzer (Dimension Xpand Plus, Siemens Healthcare, Germany), and the level of the metabolic markers were assayed by the corresponding reagent cartridges (Siemens Healthcare, Germany).

2.4. Blood Pressure Measurement and Ultrasound Examination. Each patient had two ultrasound examinations of the carotid artery. All ultrasound examinations were performed using the Esaote MyLab Twice ultrasound unit in conjunction with a 4–13 MHz linear transducer (Esaote, Genoa, Italy). Before the ultrasound examination, blood pressure of the patient was measured by a sphygmomanometer (Tensoval, Hartmann, Germany) at the left upper arm in sitting position after the patient had at least 10 minutes of rest. The systolic and diastolic pressures were then inputted into the ultrasound unit for the assessment of CAS.

The CIMT was evaluated with longitudinal scans of the CCA. CIMT was measured on the far wall over a 10 mm segment of the CCA from a point 10 mm proximal to the inferior end of the carotid bifurcation (Figure 1(a)). During the longitudinal scanning of the CCA, the transducer was slightly angled medially or laterally and rotated in clockwise or anticlockwise direction until a scan plane, which clearly demonstrated the carotid intima and media layers, was obtained. CIMT was measured using an automated quantification programme of the ultrasound unit, radiofrequency-based quality intima-media thickness (RF-QIMT) (Esaote, Genoa, Italy), which automatically identified the lumen-intima interface and media-adventitia interface of the CCA for measuring the CIMT (Figure 1(a)).

Similar to the measurement of CIMT, CAS was measured at the same segment of the CCA in the longitudinal scans. Scanning was performed carefully until a scan plane, which clearly shows the near and far walls of the CCA and demonstrated the CCA with maximum and uniform lumen diameter along the artery, was obtained. CAS was measured using an automated quantification programme of the ultrasound unit, radiofrequency-based quality arterial stiffness (RF-QAS) (Esaote, Genoa, Italy) (Figure 1(b)). The RF-QAS uses the echo-tracking technique, which tracks the movement of the near and far walls of the pulsating CCA during the scanning and measures the changes of the artery diameter during the pulsation. With the systolic and diastolic blood pressure values of the patients inputted to the ultrasound unit, the distensibility (compliance) and stiffness of the artery were automatically evaluated by the ultrasound system. In the evaluation of carotid arterial stiffness, five stiffness parameters were investigated: (1) distensibility coefficient (DC); (2) compliance coefficient (CC); (3) index α; (4) index β; and (5) pulse wave velocity (PWV). The higher α, β or PWV and the lower DC and CC are, the stiffer the carotid artery is. The stiffness parameters were calculated by the ultrasound system after the tracking of the arterial walls during the scanning.

Each carotid artery was scanned and measured three times, and the mean value of CIMT and CAS measurements were used for the data analyses. All the ultrasound examinations were performed by the same operator, and the operator was blinded to the grouping of the patients.

For the patients in the intervention group, blood tests and ultrasound examinations were performed before the commencement of the DSME programme as the baseline and one month after the completion of the DSME programme. For the patients in the control group, they had two blood tests and two ultrasound examinations with a time interval of three months.

2.5. Statistical Analysis. The continuous data was expressed as mean ± standard deviation (SD). Shapiro-Wilk test was used for checking the normality of distribution. If the data was normally distributed, t-test was used. Otherwise, nonparametric tests were applied. Demographic data and baseline characteristics between the intervention and control groups were compared using χ^2 test, t-test, or Mann-Whitney U test. Paired t-test or Wilcoxon Signed Ranks test were utilized to compare the measurements between the baseline and follow-up examinations of the patients. The differences between the intervention and control groups in the changes from the baseline to the follow-up examinations were determined using t-test or Mann-Whitney U test. All the data analyses were performed using Statistical Product and Service Solutions

Table 2: Comparison of ultrasound parameters of the carotid artery and metabolic markers between the baseline and follow-up assessments in the intervention and control groups.

	Intervention ($n = 44$)			Control ($n = 44$)		
	[#]Baseline	Follow up	P value	[#]Baseline	Follow up	P value
Age, years	58.9 ± 8.4	—	—	57.8 ± 8.2	—	—
Gender, female/male, n	22/14	—	—	28/12	—	—
Duration of diabetes, years	8.7 ± 6.9	—	—	7.3 ± 6.4	—	—
IMT, μm	702.3 ± 127.7	678.9 ± 126.2	0.025*	693.4 ± 127.3	687.1 ± 135.5	0.579
DC, 1/KPa	0.017 ± 0.009	0.016 ± 0.005	0.821	0.016 ± 0.006	0.017 ± 0.006	0.529
CC, mm^2 /KPa	0.731 ± 0.284	0.730 ± 0.266	0.660	0.753 ± 0.262	0.735 ± 0.279	0.525
α	6.081 ± 2.190	5.551 ± 1.784	0.122	5.551 ± 1.558	5.5597 ± 1.800	0.124
β	12.373 ± 4.418	11.280 ± 3.612	0.099	11.289 ± 3.148	11.400 ± 3.640	0.677
PWV, m/s	8.428 ± 1.618	8.078 ± 1.487	0.177	7.911 ± 1.177	8.028 ± 1.436	0.584
Blood glucose, mmol/L	7.689 ± 1.639	7.517 ± 1.602	0.238	7.968 ± 1.729	8.128 ± 1.771	0.427
Total cholesterol, mmol/L	4.664 ± 0.944	4.440 ± 0.961	0.034*	4.865 ± 0.879	4.550 ± 0.714	<0.001*
HDL, mmol/L	1.320 ± 0.297	1.343 ± 0.325	0.160	1.320 ± 0.337	1.281 ± 0.340	0.303
LDL, mmol/L	2.810 ± 0.768	2.590 ± 0.844	0.005*	2.927 ± 0.748	2.631 ± 0.674	<0.001*
Triglyceride, mmol/L	1.162 ± 0.644	1.112 ± 0.591	0.626	1.345 ± 0.841	1.389 ± 0.753	0.850
HbA1c, %	6.970 ± 0.915	6.772 ± 0.767	0.039*	7.038 ± 1.042	7.118 ± 1.300	0.102
Weight, kg	60.25 ± 9.54	59.06 ± 9.03	<0.001*	65.66 ± 13.38	65.05 ± 12.71	0.066
BMI, kg/m^2	23.82 ± 4.57	23.25 ± 4.14	<0.001*	25.42 ± 4.65	25.03 ± 4.35	0.019*
SBP, mmHg	127.1 ± 17.7	125.9 ± 16.1	0.692	122.5 ± 14.8	124.8 ± 17.6	0.498
DBP, mmHg	75.3 ± 9.0	75.2 ± 7.3	0.916	73.0 ± 10.1	75.1 ± 10.2	0.221

[#]No significant difference ($P > 0.05$) was found in age, gender, duration of type 2 diabetes, metabolic markers, CIMT, and CAS between the intervention and control groups in the baseline assessment.

*P value indicates significant difference between baseline and follow-up examination in either intervention group or control group. IMT: intima-media thickness; DC: distensibility coefficient; CC: compliance coefficient; PWV: pulse wave velocity; HDL: high density lipoprotein; LDL: low density lipoprotein; HbA1c: hemoglobin A1c; BMI: body mass index; SBP: systolic blood pressure; and DBP: diastolic blood pressure.

(SPSS) version 20 (IBM, Armonk, New York, United States). A P value < 0.05 was considered to be significant.

3. Results

3.1. Demographic Data. There was no significant difference in age, gender, duration of type 2 diabetes, metabolic markers, CIMT, and CAS between the intervention and control groups at the baseline assessment ($P > 0.05$, Table 2).

3.2. Metabolic Markers. For the patients in the intervention group, there was a significant decrease in the HbA1c level and body weight in the follow-up examination when compared with the baseline examination ($P < 0.05$, Table 2). However, similar observation was not found in the control group ($P > 0.05$, Table 2). The change of HbA1c and body weight after 3 month was significantly greater in the intervention group than in the control group ($P < 0.05$, Table 3).

For both intervention and control groups, there was a significant decrease in the total cholesterol, LDL, and BMI in the follow-up examination ($P < 0.05$, Table 2), but the improvements in the total cholesterol, LDL, and BMI after 3 months were not significant between the intervention and control groups ($P > 0.05$, Table 3). The blood glucose, triglyceride, HDL, SBP, and DBP levels were also not significantly different between the two study groups ($P > 0.05$, Table 3).

3.3. Atherosclerotic Parameters. In the intervention group, CIMT of the patients was significantly decreased in the follow-up examination ($P < 0.05$, Table 2). In contrast, there was no significant change in CIMT of the patients in the control group between the baseline and follow-up examinations ($P > 0.05$, Table 2). However, the difference in the change of CIMT after 3 months was not significant between the two groups ($-23.3 \pm 68.1\,\mu$m versus $-6.3 \pm 71.4\,\mu$m; $P > 0.05$; Table 3).

In both the intervention and control groups, there was no significant difference in CAS parameters (DC, CC, α, β, and PWV) between the baseline and follow-up examinations ($P > 0.05$, Table 2). The changes in these parameters after 3 months were also not significantly different between the two groups (Table 3, $P > 0.05$).

4. Discussion

The present study was a randomized and controlled clinical study which comprehensively evaluated the potential ameliorative effect of diabetes self-management education on metabolic markers and atherosclerotic parameters in patients with type 2 diabetes. In the present study, the self-management education given to the patients was of low intensity, which contained eight 2-hour sessions and encouraged the patients to follow guidelines instead of setting goals (e.g., weight loss) that patients should achieve. In spite

Table 3: Change of characteristics of subjects from baseline to follow-up assessments in the intervention and control groups.

Characteristics	Intervention $n = 36$	Control $n = 40$	P value
ΔIMT, μm	−23.3 ± 68.1	−6.3 ± 71.4	0.298
ΔDC, 1/KPa	−0.002 ± 0.01	0.001 ± 0.008	0.317
ΔCC, mm^2 /KPa	0.018 ± 0.200	−0.012 ± 0.236	0.487
Δα	−0.531 ± 2.018	0.046 ± 1.637	0.185
Δβ	−1.093 ± 4.009	0.118 ± 3.334	0.193
ΔPWV, m/s	−0.351 ± 1.530	0.117 ± 1.339	0.159
ΔBlood glucose, mmol/L	−0.172 ± 1.400	0.160 ± 0.956	0.149
ΔTotal cholesterol, mmol/L	−0.219 ± 0.546	−0.315 ± 0.527	0.351
ΔHDL, mmol/L	0.024 ± 0.161	−0.039 ± 0.173	0.596
ΔLDL, mmol/L	−0.220 ± 0.446	−0.296 ± 0.489	0.106
ΔTriglyceride, mmol/L	−0.050 ± 0.425	0.044 ± 0.583	0.643
ΔHbA1c, %	−0.200 ± 0.560	0.080 ± 0.741	0.004*
ΔWeight, kg	−1.19 ± 1.39	−0.61 ± 2.04	0.036*
ΔBMI, kg/m^2	−0.57 ± 1.00	−0.39 ± 1.00	0.105
ΔSBP, mmHg	−1.36 ± 16.70	2.34 ± 15.24	0.388
ΔDBP, mmHg	−0.05 ± 9.54	2.25 ± 9.74	0.223

*P value indicates significant difference in changes of variables between the intervention group and control groups. IMT: intima-media thickness; DC: distensibility coefficient; CC: compliance coefficient; PWV: pulse wave velocity; HDL: high density lipoprotein; LDL: low density lipoprotein; HbA1c: hemoglobin A1c; BMI: body mass index; SBP: systolic blood pressure; and DBP: diastolic blood pressure.

of the low intensity, this education enabled the patients to systematically receive the information of self-management of type 2 diabetes mellitus. Our result showed that the patients in the intervention group had significant reduction of HbA1c level and body weight after receiving the education as compared to the control group, indicating that the education had positive effects for improving the health status of patients with type 2 diabetes.

The primary outcome of the present study is improved glycemic (in terms of reduced HbA1c) and body weight control of patients after receiving the DSME. HbA1c is an important indicator showing the severity of diabetes mellitus. Stratton et al. reported that each 1% reduction of the HbA1c level was related to a 37% reduction of microvascular complications, a 21% reduction of diabetes-related death and a 14% reduction of myocardial infarction [11]. Any reduction in the HbA1c level decreases the risk of diabetes-related complications [11]. In the present study, the HbA1c level in the intervention group was significantly decreased with a mean reduction of 0.2% after receiving the self-management education. In contrast, the HbA1c level in the control group did not show significant variation. The reduction of HbA1c after 3 month was significantly greater in the intervention group than in the control group. Thus, the results of the present study showed that DSME improved HbA1c control in patients with type 2 diabetes. The finding was consistent with previous study in which the HbA1c level of the diabetic patients was significantly decreased after receiving DSME [12].

Overweight is a common complication of DM and is associated with the development of atherosclerosis. It has been reported that ≥2% of weight loss in diabetic patients could mediate significant improvement of cardiovascular risk factors [13]. The DSME programme in the present study resulted in a significant weight loss in the intervention group ($P < 0.05$, Table 2) but not in the control group ($P > 0.05$, Table 2). The change of body weight after 3 months was significantly larger in the intervention group when compared with the control group (−1.89 ± 2.23% versus −0.77 ± 2.68%; $P < 0.05$). 44.4% of patients (16 in 36) in the intervention group lost ≥2% of initial weight, whereas only 22.5% (9 in 40) of patients in the control group achieved the improvement ($P < 0.05$). For the patients who had lost ≥2% of weight ($n = 25$), they had a significant improvement of HbA1c level when compared with the patients who had not lost ≥2% of weight ($n = 51$, −0.3 ± 0.93% versus 0.07 ± 0.44%; $P < 0.05$). These results suggested that the DSME programme in the present study resulted in significant weight loss which led to significant reduction of HbA1c level.

In contrast, DSME in the present study did not improve other metabolic markers. In both the intervention and control groups, there was significant decrease in the total cholesterol and LDL of the patients in the follow-up assessment. However, the changes in the total cholesterol and LDL during the study period were not significantly different between the intervention and control groups ($P > 0.05$, Table 3). Previous studies have reported that lipid profiles increased in colder seasons but decreased in warmer seasons [14]. The present study was conducted from March to June when the temperature was rising during the study period. Thus, we speculated that the variation of these parameters may be related to their seasonal changes rather than the effect of DSME. In addition, the changes in the triglyceride, HDL, plasma glucose, SBP, and DBP levels were also not

significantly different between the two study groups ($P > 0.05$, Table 3). The intervention of the present study was in low intensity with 8 DSME lessons and had a relatively short time interval (3 months). Therefore, the effect of this DSME was only demonstrated as the change in homeostasis of HbA1c and body weight but not in the homeostasis of some other metabolic markers.

There were also no significant improvements in CIMT and CAS in the intervention group when compared with the control group ($P > 0.05$, Table 3). The negative findings in CIMT and CAS may attribute to the low intensity and short term of the intervention in the present study. Kim et al. conducted an intervention with intensive lifestyle modification which significantly decreased the progression of CIMT in type 2 diabetics [8]. In that study, the patients with type 2 diabetes in the intervention group were asked to receive a 16-lesson training involving healthy diet, exercise, and behaviors on a one-on-one training basis and to achieve goals such as reducing body weight to a certain level (5% of weight loss in obese subjects), undertaking sufficient physical activity (at least 150 min/week of brisk walking) and decreasing energy intake during a 6-month period. The intensive lifestyle modification reduced 1% of HbA1c and 40 μm of CIMT, which was greater than those in the present study. However, the one-on-one training basis is time-consuming and not cost-effective and may not be feasible for all patients. The DSME programme used in the present study, even though was in low intensity, benefited the diabetic patients by significantly decreasing their HbA1c and body weight, and it is less time-consuming and more cost effective which could be more suitable for and acceptable by the patients.

There were limitations in the present study. Firstly, the time interval of the baseline and follow-up assessments was relatively short. Therefore, possible changes of CIMT, CAS, and some metabolic markers that are related to the DSME are not demonstrated. In addition, the long-term effect of the low intensity self-management education on diabetic patients was not fully evaluated in the present study. Moreover, the sample size of the present study was small with only 36 patients in the intervention group and 40 patients in the control group. Further investigations of the long-term effect of the DSME and with a larger sample size are suggested.

As a conclusion, DSME, even though in low intensity, significantly improved the glycemic and body weight control in patients with type 2 diabetes.

Conflict of Interests

No commercial association was involved for this study. All authors declare that they have no conflict of interests regarding the data, results, and conclusions described in this study.

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Clinical Study

Diabetic Retinopathy Treated with Laser Photocoagulation and the Indirect Effect on Glycaemic Control

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Purpose. To identify any possible relation between glycaemic control and previous laser photocoagulation for diabetic retinopathy. *Methods*. Seventy-two patients with diabetes were included in the study and were separated into 2 groups according to previous treatment (group A) or not (group B) with argon laser photocoagulation. Glycaemic control was estimated by measuring blood levels of HbA1c in four consecutive measurements. *Results*. Blood levels of HbA1c in group A were significantly lower 3, 6, and 12 months after laser treatment as compared to blood levels of HbA1c before laser treatment ($7.1 \pm 0.4\%$ versus $7.6 \pm 0.9\%$, $7.2 \pm 0.2\%$ versus $7.6 \pm 0.9\%$, and $7.1 \pm 0.2\%$ versus $7.6 \pm 0.9\%$, resp., all $P < 0.05$). Blood levels of HbA1c in group B did not differ significantly in four consecutive measurements. *Conclusion*. Our results suggest that we should anticipate a better glycaemic control in cases of patients with diabetes previously treated with laser photocoagulation.

1. Introduction

Diabetic retinopathy (DR) is the most common microvascular complication of diabetes, which can result even in blindness [1]. DR can be classified into two categories: nonproliferative (NPDR) and proliferative (PDR) [2]. PDR occurs with severe retinal ischemia and is characterized by the growth of new blood vessels on the optic disc or elsewhere in the retina [2]. Diabetic macular edema (DME) can occur at any stage of DR and is regarded as the principal cause of vision loss in patients with diabetes [2]. Retinal hypoxia is implicated in the pathogenesis of DME and in the development of retinal neovascularization. Hypoxia results in increased expression of vascular endothelial growth factor (VEGF), which is the most potent inducer of increased vascular permeability and the trigger for the formation of abnormal and leaking new vessels [3]. In previously published studies we have already shown the well-established involvement of vascular endothelial growth factor (VEGF) and the contribution of other growth factors [4] in the pathogenesis of PDR [5] and NPDR with DME [3].

Clinical trials have also shown the effectiveness of laser photocoagulation, vitrectomy, and control of hyperglycemia and hypertension for DR [1]. In this study we examined the hypothesis that previous treatment with argon laser photocoagulation in patients with diabetes is positively related to their glycaemic control.

2. Patients and Methods

The study was conducted prospectively in 2009-2010 at the Aristotle University of Thessaloniki, Greece, following the tenets of the Declaration of Helsinki. Approval of the Institutional Review Board Ethics Committee of the Medical School of Aristotle University of Thessaloniki was also obtained. All patients signed an informed consent after the purpose of the study was explained in detail to each subject.

Patients with diabetes were included in the study and were separated into 2 groups according to treatment (group A) or not (group B) with argon laser photocoagulation. Inclusion criteria for both groups included (i) patients with type 2 diabetes mellitus, (ii) visual acuity ranging between 20/40 and 20/70 for each eye of the same subject, (iii) NPDR with DME diagnosed with biomicroscopy and confirmed on fundus fluorescein angiography (FFA). Exclusion criteria for both groups included (i) previous treatment with laser photocoagulation, (ii) no evidence of PDR or clinically significant macular edema (CSME) on biomicroscopy or FFA.

All patients with diabetes included in the study were offered laser treatment after receiving counseling by the same retinal specialist related to their management of DR and were provided information leaflet regarding the laser treatment and were treated in the same hospital by the same retinal specialist. Glycaemic control was estimated by measuring blood levels of HbA1c (Hemoglobin A1c) in four consecutive measurements, that is, at baseline and at 3 months, 6 months, and 12 months after laser treatment in group A and in four consecutive measurements at baseline and at 3, 6, and 12 months in group B as well.

All values were expressed as the mean ± standard error (mean ± SE). Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS). The differences between groups were analyzed by multivariate analysis of variance (MANOVA). The differences between groups with respect to sex were tested by chi-square test (Table 1). A two-tailed P value of less than 0.05 was considered to indicate statistical significance.

3. Results

Seventy-two patients with diabetes were included in the study: group A (treated with laser) 36 patients and group B (untreated) 36 patients. Characteristics of patients included in the study are shown in Table 1. There were no significant differences between the two diabetic groups with respect to sex, age, duration of diabetic disease, and blood levels of HbA1c prior to treatment with argon laser photocoagulation.

Furthermore, there were also no significant differences between the two groups with respect to comorbidities such as systemic arterial hypertension (SAH), dyslipidemia, renal dysfunction and atherosclerotic cardiovascular disease, their income, educational level, habits (smoking and/or drinking), and insurance status (private versus social).

Blood levels of HbA1c in group A were significantly lower 3 months after laser treatment as compared to blood levels of HbA1c before laser treatment (7.1 ± 0.4% versus 7.6 ± 0.9%, $P < 0.05$, MANOVA). Interestingly, blood levels of HbA1c in group A sustained significantly decreased after 6 months after laser treatment as compared to blood levels of HbA1c before laser treatment (7.2 ± 0.2% versus 7.6 ± 0.9%, $P < 0.05$, MANOVA). More interestingly, blood levels of HbA1c in group A sustained significantly decreased even after 12 months after laser treatment as compared to blood levels of HbA1c before laser treatment (7.1 ± 0.2% versus 7.6 ± 0.9%, $P < 0.05$, MANOVA). No significant negative correlations between the extent or the type (focal, grid, or both) of laser photocoagulation and the concentration of HbA1c in blood after laser treatment were found in group A (all P values > 0.05). Blood levels of HbA1c in group B did not differ significantly in four consecutive measurements (7.5 ± 0.8% versus 7.6 ± 0.9%, 7.4 ± 0.8% versus 7.6 ± 0.9%, and 7.4 ± 0.6% versus 7.6 ± 0.9%, resp., all $P > 0.05$) (Table 2).

Table 1: Baseline characteristics (mean ± standard error) of patients with diabetes included in the study.

	Group A (treated)	Group B (untreated)
Total patients (n)	36	36
Age (yrs)	62 ± 2.5	65 ± 1.8
Duration of diabetes (yrs)	14 ± 3	14 ± 1.5
HbA1c (%) at baseline (g/dL)	7.6 ± 0.9	7.6 ± 0.9
Systolic BP (mmHg)	165 ± 9	160 ± 11
Weight (Kg)	82 ± 10	80 ± 12
BMI (Kg/m^2)	32 ± 6	32 ± 4

No significant positive correlations were found between blood pressure (BP) levels, body mass index (BMI), weight, and the laser treatment. But there were positive correlations between some social habits, such as smoking, alcohol, exercise, and the laser treatment but not significant ones.

4. Discussion

Panretinal (PRP) and focal or grid laser photocoagulation, when indicated in patients with PDR or DME, respectively, has beneficial effect on DR by reducing the risk of severe visual loss even more than 50% [6–8]. In our study, we came to an interesting observation that previous argon laser photocoagulation in patients with diabetes is positively related to their glycaemic control. A feasible explanation for this trend is that patients with diabetes who undergo laser photocoagulation treatment apparently consider laser photocoagulation as an operation and subsequently attain the attitude of the operated patient, who in general complies better to doctor's guidelines, such as antidiabetic treatment, exercise and diet, achieving stricter glycaemic control, and hypothesis that was generally supported from our Institutional Psychologist.

Additionally, the improved glycaemic control could be attributed to a psychological effect of becoming motivated to improve the health status [9] after having been subjected to laser photocoagulation and a stark realization of the diabetic disease that is leading to impaired eyesight. With the inherent fear of vision loss in this patient population some had probably experienced some degree of visual loss and may fear losing more, which could motivate them to change their behaviour. Also the discomfort or pain associated with the procedure [10] could be a potential motivator to alter diet to prevent the likelihood of requiring further courses of treatment.

On the other hand, patients with diabetes who reject the laser treatment obviously do not realize the seriousness of their eye involvement (either as a result of their own

Table 2: HbA1c (g/dL) blood levels (mean ± SE) in both groups at baseline and at 3 consecutive measurements after laser treatment.

	Group A (treated)	Group B (untreated)
Baseline	7.6 ± 0.9%	7.6 ± 0.9%
Month 3	7.1 ± 0.4%	7.5 ± 0.8%
Month 6	7.2 ± 0.2%	7.4 ± 0.8%
Month 12	7.1 ± 0.2%	7.4 ± 0.6%

perception or because of a patient-doctor communication failure) and thus continue to their previous way of living (in terms of glycaemic control, systemic follow-ups with their diabetologists, healthy eating).

We also suggest that diabetologists have an additional reason to encourage patients with diabetes to visit an ophthalmologist [11], expect for direct treatment of DR [3, 6, 8]; they should expect better glycaemic control in certain cases. We are not aware if the same results for better glycaemic control imply for patients who had laser for PDR; to our experience, because PDR does not always imply severe visual loss, patients with PDR do not always realize the seriousness of DR eye disease.

5. Conclusion

To our knowledge, this is the first study that examines the possible "indirect effect" of laser treatment to glycaemic control. Larger number of patients and duration of glycaemic control could safely examine more related parameters and support our findings.

Conflict of Interests

None of the authors have any financial support/any proprietary interests or conflicts of interest related to this submission.

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Research Article

Acarbose Treatment and the Risk of Cardiovascular Disease in Type 2 Diabetic Patients: A Nationwide Seven-Year Follow-Up Study

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Objective. To investigate the potential benefits of acarbose treatment on cardiovascular disease (CVD) in patients with type 2 diabetes by using nationwide insurance claim dataset. *Research Design and Methods*. Among 644,792 newly diagnosed type 2 diabetic patients without preexisting CVD in a nationwide cohort study, 109,139 (16.9%) who had received acarbose treatment were analyzed for CVD risk. Those with CVD followed by acarbose therapy were also subjected to analysis. *Result*. During 7 years of follow-up, 5,081 patients (4.7%) developed CVD. The crude hazard ratio (HR) and adjusted HR were 0.66 and 0.99, respectively. The adjusted HR of CVD was 1.19, 0.70, and 0.38 when the duration of acarbose use was <12 months, 12–24 months, and >24 months, respectively. Adjusted HR was 1.14, 0.64, and 0.41 with acarbose cumulative doses <54,750 mg, 54,751 to 109,500 mg, and >109,500 mg, respectively. *Conclusion*. In patients with type 2 diabetes without preexisting CVD, treatment with acarbose showed a transient increase in incidence of CVD in the initial 12 months followed by significant reductions of CVD in prolonged acarbose users. After the first CVD events, continuous use of acarbose revealed neutral effect within the first 12 months. The underlying mechanisms require further investigations.

1. Introduction

Previous observational studies have indicated that postprandial hyperglycemia seems to play a unique role in the development of cardiovascular complications in patients with type 2 diabetes [1–4]. This is particularly relevant for Asian diabetic subjects whose postprandial hyperglycemia contributes more prominently to the level of HbA1c [5].

Acarbose, an oral antidiabetic agent, competitively inhibits the alpha-glucosidase in the brush-border of the small intestine and delays the digestion of complex carbohydrates in the upper small bowel that subsequently retards the absorption of glucose and blunts the postprandial hyperglycemia [6, 7]. Compared to metformin, acarbose is similar in efficacy to initial therapy in Chinese patients newly diagnosed with type 2 diabetes [8]. Results from the STOP

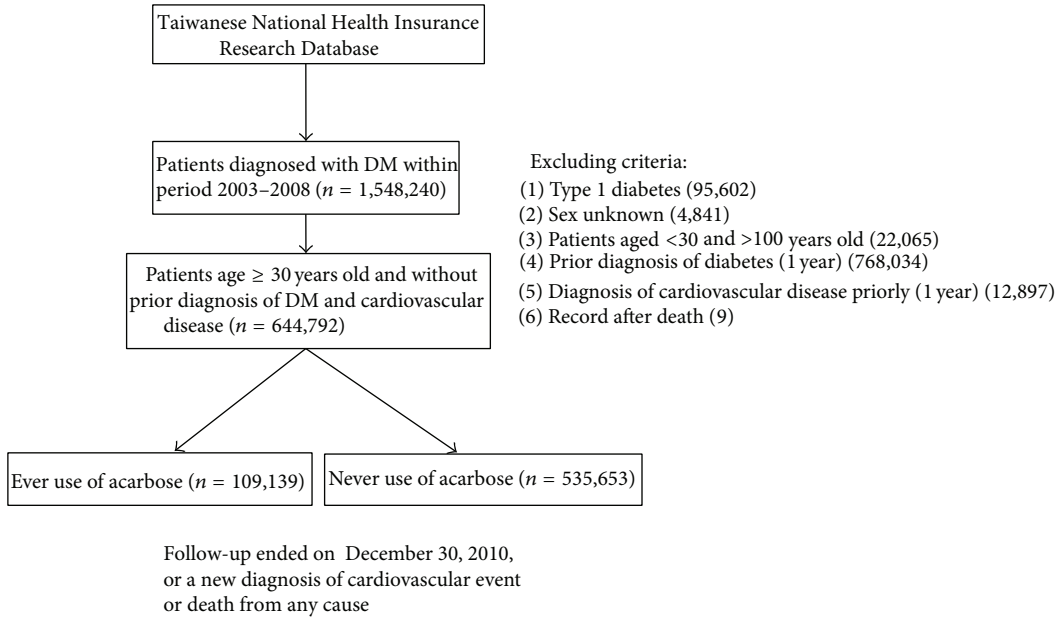

Figure 1: Study subjects' selection flow chart in those newly diagnosed with diabetes who do not have preexisting CVD.

NIDDM (Study to Prevent Non-Insulin-Dependent Diabetes Mellitus) had shown that acarbose treatment was associated with a 49% risk reduction in cardiovascular events in subjects with impaired glucose tolerance [9]. Even after adjusting for major risk factors, the reduction in the risk of cardiovascular events and hypertension associated with acarbose treatment was still statistically significant. In a meta-analysis of patients with type 2 diabetes, acarbose use was associated with a 35% reduction in cardiovascular events [10]. The mechanisms by which acarbose lowers the risk of cardiovascular events is putatively attributed to the diminution in postprandial hyperglycemic excursion and thus leads to a lowering of the impact of oxidative stress and tissues protection as well as a series of benefits [6, 11].

However, the above-mentioned findings were either limited to use in subjects with IGT (impaired glucose tolerance) [9], short-term studies [11], or meta-analysis [12]. Currently, there are no population-based long-term investigations about the potential cardiovascular effect of acarbose treatment in patients with type 2 diabetes. To address this issue, we used the Taiwanese National Health Insurance Research Database (NHIRD) to evaluate the cardiovascular effect of acarbose treatment in newly type 2 diabetic patients without previous CVD [13]. We also examined the effects of continuous use of acarbose on recurrent CVD in those diabetic subjects who had first cardiovascular events.

2. Research Design and Methods

2.1. Data Source. Since March 1, 1995, Taiwan had launched a single-payer National Health Insurance Program.

And in 2007, 98% of Taiwan's populations had been enrolled in this program [13]. Data in the National Health Insurance Research Database (NHIRD) that could be used to identify patients or care providers is scrambled before being sent for database construction. The database is further scrambled before being released to researchers [13].

2.2. Methods. In this study, diabetic patients were identified using the ICD-9-CM code (250) with at least 3 outpatient visits or hospitalization treatment once which has been validated. Diabetic subjects were enrolled if they fulfilled the following three criteria: (1) diagnosed with diabetes between year 2003 and 2008; (2) age older than 30 years and less than 100 years as of January 1, 2003; and (3) had no diagnosis of cardiovascular diseases before year 2003 or one year prior to the diagnosis of diabetes.

Patients with type 1 diabetes, having diagnosis of diabetes in prior one year, and with sex unknown in the data were all excluded. The primary endpoint is cardiovascular diseases described below. The follow-up period started from Jan. 1, 2003 and lasted until Dec. 31, 2010 (Figure 1).

Cardiovascular outcomes were identified with the diagnosis of cardiovascular disease (coronary heart disease with ICD-9-CM codes 410–414, stroke with ICD-9CM codes 430–438, and peripheral arterial occlusive disease (PAOD) with ICD-9CM codes 443.9). To confirm the diagnosis, all above events required hospitalization for at least once.

The comorbidities, including hypertension (ICD-9CM codes 401–405), chronic kidney disease (CKD) (ICD-9CM codes 582-583), and hyperlipidemia (ICD-9CM codes 272), were recorded with at least 3 times of outpatient visit or admission once.

Using the NHIRD, a total of 644,792 newly diagnosed type 2 diabetic patients were enrolled. Among them 109,139 had received acarbose treatment during the period of follow-up. The follow-up period ended on December 30, 2010 or when a newly diagnosed cardiovascular event or death from any cause developed (Figure 1 and Table 1).

Table 1: Demographics, comorbidities, and concomitant use of antidiabetic medications in those acarbose users with (n = 5081) or without (104058) CVD development.

Characteristic	Overall number 109,139	Did not develop CVD 104,058	Developed CVD 5,081	P value
Age, years (mean ± SD)				
30–39	9,417 (8.6)	9,239 (8.9)	178 (3.5)	
40–49	25,707 (23.6)	24,936 (24.0)	771 (15.2)	
50–59	32,921 (30.2)	31,496 (30.3)	1,425 (28.0)	
60–69	22,343 (20.5)	21,035 (20.2)	1,308 (25.7)	<0.0001
70–79	14,295 (13.1)	13,269 (12.8)	1,026 (20.2)	
80–89	4,152 (3.8)	3,805 (3.7)	347 (6.8)	
>=90	304 (0.3)	278 (0.3)	26 (0.5)	
Sex				
Male	59,473 (54.5)	56,033 (53.8)	3,440 (67.7)	<0.0001
Female	49,666 (45.5)	48,025 (46.2)	1,641 (32.3)	
Comorbidities				
Hypertension	81,341 (74.5)	76,944 (73.9)	4,397 (86.5)	<0.0001
Hyperlipidemia	70,106 (64.2)	67,213 (64.6)	2,893 (56.9)	<0.0001
CKD	6,965 (6.4)	6,348 (6.1)	617 (12.1)	<0.0001
Other diabetes medications				
Rosiglitazone	9,420 (8.6)	9,010 (8.7)	410 (8.0)	<0.0001
Metformin	72,378 (66.3)	70,037 (67.3)	2,341 (46.1)	<0.0001
Pioglitazone	14,963 (13.7)	14,557 (14.0)	406 (8.0)	<0.0001
Sulfonylurea	51,765 (47.4)	49,915 (48.0)	1,850 (36.4)	<0.0001
Meglitinide	13,161 (12.1)	12,551 (12.1)	610 (12.0)	<0.0001
Insulin	8,994 (8.2)	8,426 (8.1)	568 (11.2)	<0.0001
DPP4 inhibitor	11,641 (10.7)	11,528 (11.1)	113 (2.2)	<0.0001

CVD: cardiovascular disease. DPP4: dipeptidyl peptidase 4. CKD: chronic kidney disease.

Cumulative duration of taking acarbose was calculated by counting the number of days between prescriptions. If the next prescription was filled within 30 days of the expected end date of the previous prescription, we assumed that therapy was uninterrupted. However, if there were no refills within the 30 days after the expected end date of the previous prescription, we assumed a gap in therapy starting 30 days after the date that the previous prescription should have ended. The cumulative duration variable was a time-varying sum of all periods of exposure even if there were gaps in treatment. Cumulative dose of acarbose was calculated in a similar fashion.

Among the 5081 patients who had received acarbose and developed CVD, 456 patients who died in the first month were excluded. A total of 4625 patients were enrolled in this study and followed up to record their recurrent CVD events. Among them, 1756 patients kept acarbose therapy while 2869 patients stopped receiving acarbose and switched to other antidiabetic regimens (see Supplemental Figure 1s of the Supplementary Material available online at http://dx.doi.org/10.1155/2014/812628).

The protocols had been submitted to the Tungs' Taichung MetroHarbor Hospital IRB (institutional review board) for review and approval was obtained.

2.3. Statistical Analysis. Distributions of subjects with and without use of acarbose according to age, gender, clinical comorbidities, and concomitant use of other diabetes medications were examined using χ^2-tests for categorical variables and Student's t-tests for continuous variables. The crude, age, gender, use of other diabetes medications, Charlson-Deyo comorbidity index (CCI), adjusted hazard ratio, and 95% confidence interval were calculated in subjects with and without use of acarbose, respectively. All analyses were performed using the SAS software, version 9.2 (SAS Institute, Cary, NC, USA).

3. Results

Among 644,792 patients with newly diagnosed type 2 diabetes identified during 2003 to 2008, 109,139 (16.9%) were treated with acarbose, either as monotherapy or in combination with other antidiabetic regimens. As compared with nonusers of acarbose, the acarbose users were younger, slightly female predominant, had more comorbidities, and were more likely to take combination treatment with other antidiabetic regimens. During 7 years (median 3.8 years) of follow-up, 5081 patients (4.7%) who had received acarbose treatment developed CVD as compared to 33,203 patients

Table 2: Crude and adjusted hazard ratio of CVD with or without acarbose use.

Risk factor	Developed CVD	Did not develop CVD	Crude				Adjusted			
			HR	95% CI		P	HR	95% CI		P
				Low	Up			Low	Up	
Never use of acarbose	33203	502450	Ref.	—	—		Ref.	—	—	—
Ever use of acarbose	5081	104058	0.66	0.641	0.680	<0.001	0.99	0.958	1.019	0.443
Cumulative duration of therapy (months)										
<12	4216	69976	0.83	0.802	0.855	<0.001	1.19	1.152	1.231	<0.001
12–24	545	17345	0.42	0.387	0.459	<0.001	0.70	0.643	0.762	<0.001
>24	320	16737	0.24	0.217	0.271	<0.001	0.38	0.341	0.425	<0.001
Cumulative dose (mg)										
1–54,750	4373	75757	0.79	0.769	0.819	<0.001	1.14	1.105	1.18	<0.001
54,751–109,500	449	15273	0.39	0.357	0.43	<0.001	0.64	0.583	0.704	<0.001
>109,500	259	13028	0.25	0.219	0.28	<0.001	0.41	0.360	0.460	<0.001

Adjusted age, gender, other diabetic medications, and CCI. CVD: cardiovascular disease.

Table 3: HR of recurrent CVD by keep or stop using acarbose.

Risk factor	Recurrence of CVD	No recurrence of CVD	Crude				Adjusted			
			HR	95% CI		P	HR	95% CI		P
				Low	Up			Low	Up	
Stop acarbose users	627	2242	Ref.	—	—		Ref.	—	—	—
Keep acarbose users	582	1174	0.84	0.752	0.944	0.003	0.88	0.781	0.987	0.029
Cumulative duration of therapy (months)										
<12	508	1142	0.98	0.87	1.1	0.715	1.00	0.891	1.132	0.947
12–24	49	18	0.52	0.392	0.702	<0.001	0.48	0.355	0.645	<0.001
>24	25	14	0.29	0.196	0.441	<0.001	0.38	0.249	0.567	<0.001
Cumulative dose (mg)										
1–10500	523	1149	0.95	0.848	1.07	0.413	0.97	0.86	1.091	0.600
10501–28000	39	16	0.45	0.324	0.621	<0.001	0.47	0.338	0.657	<0.001
>28000	20	9	0.33	0.211	0.518	<0.001	0.40	0.253	0.628	<0.001

Adjusted age, gender, other diabetic medications, and CCI. CVD: cardiovascular disease.

(6.2%) who never took acarbose therapy. Furthermore, 2619 patients (2.4%) who had received acarbose treatment developed stroke as compared to 18212 (3.4%) who never took acarbose therapy. The study showed that acarbose users group had a lower risk of developing CVD ($P < 0.001$) and stroke ($P < 0.037$) (Supplemental Table 1s). Table 1 depicted data of those with or without CVD in acarbose users. Those with CVD were older, male predominant, had more comorbidities with hypertension and CKD but were less likely to have hyperlipidemia (all $P < 0.001$), and had lesser use of combined oral hypoglycemic agents but more use of insulin as compared to those without CVD (Table 1).

By using those without exposure to acarbose as reference value, the crude (95% CI) hazard ratio (HR) of acarbose users who developed CVD was 0.66 (95% CI 0.641–0.680, $P < 0.001$) (Table 2). After adjusting for age, gender, Charlson-Deyo comorbidity index (CCI), and concomitant use of other antidiabetic regimens, the adjusted HR became 0.99 (0.958 to 1.019, $P = 0.443$). Further analysis indicated that adjusted HR of CVD was 1.19 (95% CI 1.152–1.231 $P < 0.001$), 0.70 (95% CI 0.643–0.762 $P < 0.001$), and 0.38 (95% CI 0.341–0.425 $P < 0.001$) when the duration of acarbose use to CVD events was <12 months, 12–24 months and >24 months, respectively. Adjusted HR was 1.14 (95% CI 1.105–1.18 $P < 0.001$), 0.64 (95% CI 0.583–0.704 $P < 0.001$), and 0.41 (95% CI 0.360-0.460 $P < 0.001$) with acarbose cumulative dose <54,750 mg, 54,751 to 109,500 mg, and >109,500 mg, respectively (Table 2).

For patients who had first event of CVD, continuous use of acarbose revealed neutral adjusted HR 1.00 (95% CI 0.891–1.132 $P = 0.947$) for recurrent CVD in those with use duration <12 months followed by lower adjusted HR in those with prolonged acarbose use as compared to those who stopped using acarbose (Table 3). Further analysis, based on cumulative dosages, showed a similar trend (Table 3).

4. Discussion

The main findings from the present dataset indicated that use of acarbose, either as monotherapy or in combination with other antidiabetic regimens, in newly diagnosed type 2 diabetes subjects who did not have preexisting CVD, provided a unique impact on the subsequent development of CVD. Specifically, use of acarbose increased the chances of developing CVD in the first 12 months of exposure. However,

the benefits on CVD began to emerge in continuous users of acarbose. Analysis of dosages of acarbose showed similar patterns.

Results from previous observational studies have demonstrated that elevated postprandial glycemic excursion contributes to increase in the risk of developing coronary heart disease or a CV event [14, 15]. By reducing postprandial hyperglycemia, the potential benefits derived from acarbose treatment have included reduced body weight [9, 12], reduced blood pressure both systolic and diastolic [9, 10, 16, 17], reduced triglyceride [18], reduced post meal lipemia [19–21], reduced postmeal activation of coagulation [22], reduced insulin resistance [18, 23], reduced carotid intima-medial thickness [24], reduced myocardial infarct size [25], marked decrease in the plasma levels of plasminogen activator inhibitor 1 and fibrinogen, and reduction in the plasma levels of oxidized low-density lipoprotein [26]. In fact, findings from STOP NIDDM [9] in subjects with IGT and results from MeRIA study [12] all support the potential benefits of acarbose management on CVD.

The exact causes of our unanticipated findings that acarbose increased CVD in the first 12 months of exposure while providing protection in those prolonged users are not clear at this moment. Our target diabetic subjects were newly diagnosed with diabetes without preexisting CVD which was different from the subjects of STOP NIDDM [1] and MeRIA [12]. Considering the time needed to form atherosclerosis, it is not reasonable to speculate that the use of acarbose enhanced occurrence of CVD events. In this regard, there has also no evidence that acarbose could precipitate rupture of those preexisting vascular plaques in acarbose users. Actually, recent study indicated that the use of acarbose could rapidly improve carotid plaque echolucency within 1 month of therapy in type 2 diabetes patients with acute coronary syndrome [27]. On the other hand, diabetic subjects who used acarbose for a prolonged period or who reached certain amount of doses showed benefits of protection, both in first CVD or recurrent CVD events. The exact mechanisms definitely require further investigations.

There are several limitations in our study. First, a causal association between acarbose and CVD cannot be ascertained based on the observational data. We cannot retrieve clinical data like lipids and glycemic, blood pressure control from this claim dataset. Although we have adjusted potential confounding, there may be other measured and unmeasured factors which we were not able to detect or obtain. The diagnosis of type 2 diabetes or the cardiovascular events according to the ICD-9-CM may also lead to some distortion.

5. Conclusions

Our study suggests that treatment with acarbose in the newly diagnosed type 2 diabetic patients who do not have preexisting CVD showed an initial increased HR followed by benefits on subsequent CVD. After the first CVD events, continuous use of acarbose revealed a relatively similar pattern of benefits on developing recurrent CVD. The effects of acarbose on CVD remain speculative before the final results of other large prospective studies come out (such as the ongoing Acarbose Cardiovascular Evaluation Trial) [28].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jui-Ming Chen and Wayne H.-H. Sheu conceived and designed the experiments. Jui-Ming Chen, Jorng-Tzong Horng, and Cheng-Wei Chang performed the experiments. Jui-Ming Chen, Wayne H.-H. Sheu, Cheng-Wei Chang, and Ying-Chieh Lin analyzed the data. Jorng-Tzong Horng contributed to reagents/materials/analysis tools. Jui-Ming Chen, Wayne H.-H. Sheu, and Cheng-Wei Chang wrote the paper and contributed equally to this work.

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Clinical Study

Relationship between HgbA1c and Myocardial Blood Flow Reserve in Patients with Type 2 Diabetes Mellitus: Noninvasive Assessment Using Real-Time Myocardial Perfusion Echocardiography

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To study the relationship between glycosylated hemoglobin (HgbA1c) and myocardial perfusion in type 2 diabetes mellitus (T2DM) patients, we prospectively enrolled 24 patients with known or suspected coronary artery disease (CAD) who underwent adenosine stress by real-time myocardial perfusion echocardiography (RTMPE). HgbA1c was measured at time of RTMPE. Microbubble velocity ($\beta\,\text{min}^{-1}$), myocardial blood flow (MBF, mL/min/g), and myocardial blood flow reserve (MBFR) were quantified. Quantitative MCE analysis was feasible in all patients (272/384 segments, 71%). Those with HgbA1c > 7.1% had significantly lower β_{reserve} and MBFR than those with HgbA1c ≤ 7.1% ($P < 0.05$). In patients with suspected CAD, there was a significant inverse correlation between MBFR and HgbA1c ($r = -0.279$, $P = 0.01$); however, in those with known CAD, this relationship was not significant ($r = -0.117$, $P = 0.129$). Using a MBFR cutoff value > 2 as normal, HgbA1c > 7.1% significantly increased the risk for abnormal MBFR, (adjusted odds ratio: 1.92, 95% CI: 1.12–3.35, $P = 0.02$). Optimal glycemic control is associated with preservation of MBFR as determined by RTMPE, in T2DM patients at risk for CAD.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a known risk factor of coronary artery disease (CAD). Cardiovascular disease is the leading cause of death in T2DM patients. Antecedent to and associated with epicardial coronary artery stenosis, T2DM patients develop abnormal microvascular function in systemic circulatory beds, including those of the myocardium [1–4]. Glycosylated hemoglobin (HgbA1c) has been established as a risk factor for T2DM patients developing microvascular atherosclerosis [5]. However, the relationship between HgbA1c, coronary artery disease (CAD), and coronary perfusion in T2DM patients has not yet been clarified.

HgbA1c level is utilized clinically as an indicator of the adequacy of glycemic control over several months prior to testing. Thus, it is felt to reflect the effectiveness of long-term glucose control in diabetes patients. The American Diabetes Association has recommended that an HgbA1c breakpoint of 7% would realize the greatest cardiovascular benefit [6]. Several studies have shown that HgbA1c is associated with the severity and progression of coronary atherosclerosis [7–9]. The risk of microvascular complications rises exponentially rather than linearly as HgbA1c increases. Conversely, each 1% reduction in HgbA1c has been shown to be associated with a 37% decrease in risk for microvascular complications and a 21% decrease in the risk of any end point or death related to diabetes [10].

The coronary system can be viewed as having two vascular parts [11]. One is composed of the larger epicardial coronary arteries, having diameters of several millimeters (up to 400 μm), and termed "conductive vessels," with low resistance to blood flow. The coronary arteries then branch into smaller arterioles (resistance vessels), and then branch yet again and again, to end in the smallest branches, comprising the second and largest part of the myocardial circulation, the capillaries, having the smallest diameters (<100 μm). Atherosclerosis in T2DM can occur both within the epicardial and microvascular circulation. However, diffuse microvascular atherosclerosis observed in T2DM is likely far more significant as it affects multiple vascular beds within the body, impacting upon diabetic complications and overall survival. Yet, microvascular disease in T2DM remains difficult to detect and document, generally requiring invasive methodology, and thus is infrequently performed.

Real-time myocardial perfusion echocardiography (RTMPE) is a noninvasive method to evaluate microcircula -tory perfusion by depletion (destruction) of microbubbles and their observed replenishment into the myocardium. By videodensitometric analysis of these refill/replenishment cycles, RTMPE can quantitatively measure myocardial perfusion parameters including microbubble velocity β (min^{-1}), myocardial blood flow (MBF, mL/min/g), and myocardial blood flow reserve (MBFR). We have previously shown that T2DM patients with known or suspected CAD have impaired RTMPE-derived quantitative myocardial perfusion parameters compared to nondiabetic patients during adenosine vasodilator stress [12].

In the current study, our aim was to determine if there was relationship between the HgbA1c level and quantitative myocardial perfusion parameters in T2DM patients with known or suspected CAD, and to determine if the HgbA1c level was an independent risk factor for prediction of myocardial perfusion status.

2. Methods

2.1. Study Population. We prospectively enrolled 24 T2DM patients (16 male; mean age: 66 ± 12 yrs.) with known or suspected CAD. The mean HgbA1c level was 7.1 ± 1.4% (range 5.4–10.9%), fasting plasma glucose was 151.6 ± 61.2 mg/dL (range 80–289 mg/dL), and duration of diabetes was 8 ± 5.1 years (range 2–25 years). Eleven patients were receiving oral hypoglycemic therapy, twelve were on insulin treatment, and one was on diet control. Patients were classified into 2 groups based on the sampled population mean threshold HgbA1c of 7.1%, which coincided with the recommended goal by the American Diabetes [6]: "Poorly controlled" = Group 1: HgbA1c level > 7.1%, and "Well-controlled" = Group 2: HgbA1c level ≤ 7.1%. Exclusion criteria included age < 18 years, moderate to severe valvular heart disease, congenital heart disease, heart failure, or contraindications to echocardiographic contrast agent or adenosine. The study was approved by the Mayo Clinic Internal Review Board, and all patients gave informed consent.

2.2. Imaging Protocol. Rest and stress RTMPE were performed using SONOS 7500 or iE33 (Philips Healthcare, Andover, MA, USA) ultrasound equipment. Definity (Lantheus Medical Imaging; North Billerica, MA, USA) 1.3 mL diluted in 60 cc 0.9% saline was infused continuously at 200 mL/hr. Definity infusion started 1 minute before RTMPE acquisition at rest and was kept constant throughout the study. Stress RTMPE images were continuously acquired after 3 minutes of adenosine infusion (140 μg/kg/min) and completed within 1 minute after discontinuation of the 6-minute adenosine infusion. Apical -4, -3, and -2 chambers and short axis views were acquired using the power modulation setting at a mechanical index (MI) of ≤ 0.2, frame rate of approximately 20 Hz, and transmit focus optimally adjusted at mitral valve level. Depletion-replenishment imaging was used with a transient, high-MI (1.2) to deplete myocardial microbubbles completely (for approximately 10–15 frames), and then replenishment was observed over 15 cardiac cycles. Images were stored digitally for offline analysis.

2.3. Image Interpretation. Quantitative RTMPE analysis was performed offline by a single observer (SSA) blinded to patient clinic data and image stage (rest versus stress). Images were evaluated from end-systolic frames using QLAB, version 5.0 (Philips Healthcare, Andover, MA, USA). According to the standard 16-segment myocardial region model, segmental regions of interest (ROI) were placed and tracked manually within the myocardium and in the adjacent left ventricular cavity at end-systolic frames. With regard to the replenishment curve parameters, A (dB) represents the plateau acoustic intensity reflecting microvascular cross-sectional area or myocardial blood volume and β (min^{-1}) represents the rate of rise of acoustic intensity increase reflecting microbubble velocity; thus, the product $A \times \beta$ is a semiquantitative estimate of MBF stress [13]. However, myocardial blood volume reflected by A is dependent on the ultrasound microbubble agent, scanner settings, and acoustic tissue properties, and it may vary within and between myocardial regions stress [14]. Therefore, we used absolute MBF (mL/min/g) to assess myocardial perfusion. The methodology and terminology for these calculations, described in detail above, and in our previous quantitative and qualitative RTMPE publications and seminal work by Vogel et al. [12, 14], are summarized as follows: absolute MBF = rBV × β/Pt, where rBV (relative blood volume) = A/A_{LV}, A_{LV} (dB) is the adjacent left ventricular videointensity in the near wall cavity and Pt is the myocardial tissue density (1.05 g/mL). Thus, "absolute" MBF = $(A/A_{\text{LV}}) \times \beta/\text{Pt}$. Reserve values were calculated as the ratio of hyperemic to baseline values of MBF.

2.4. Statistical Analysis. Continuous data were reported as mean ± standard deviation or median (25% IQR, 75% IQR). Frequencies were used to report categorical variables and were compared using the chi-square test or Fisher's exact test accordingly. RTMPE feasibility was evaluated by reporting the percentage of analyzable myocardial segments. Wilcoxon sign rank test was used to compare quantitative RTMPE variables before and after adenosine stress. Wilcoxon rank

sum test was used to compare quantitative RTMPE variables between two groups. The correlation between HgbA1c and RTMPE parameters was assessed with Spearman rank correlation. Univariate and multivariate logistic regression analysis was used to evaluate the HgbA1c as a risk factor for decreased MBF relative to traditional risk factors. Variables with likelihood test P value < 0.2 in univariate analysis were included in the multivariate logistic regression model and provided final adjusted odds ratios and 95% confidence intervals. In all analyses the significance level was set at two-tailed $P < 0.05$. All analyses were performed using JMP version 9.0 (SAS Campus Drive, Cary, NC, USA).

3. Results

3.1. Clinical Data. There were 9 patients in Group 1 (poorly controlled) and 15 patients in Group 2 (well-controlled). Baseline clinical characteristics of the study groups are summarized in Table 1. There were no significant differences in clinical characteristics between two groups. Similarly, hemodynamic recordings at rest or during vasodilator stress did not differ significantly between two groups. Overall, adenosine administration resulted in significant increase (mean ± SE) in both heart rate [10.0 ± 2.1 bpm, ($P < 0.0001$)] and rate-pressure product [876.54 ± 391 bpm × mmHg ($P < 0.03$)]. The majority of patients were taking insulin and/or oral hypoglycemic drugs. The treatment details (% of study population) are shown in Figure 1, along with the mean HgbA1c level for each treatment group.

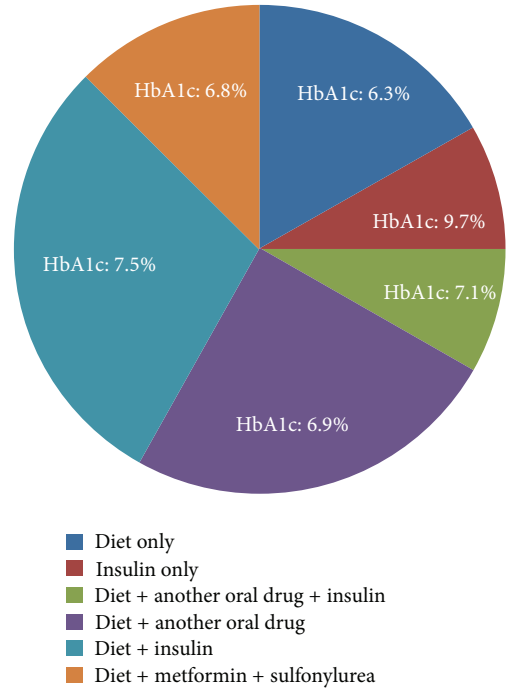

Figure 1: Proportion of study population receiving diabetes treatments and mean HgbA1C for each treatment group.

3.2. Quantitative MCE. Quantitative MCE parameter measurements were feasible in all 24 patients. Of 384 total segments, 272 segments (71%) were analyzable both at rest and peak, in order to derive the MBF reserve value. Inability to perform quantitative MCE analysis was related to failure of curve fitting algorithm or lack of complete transmural visualization of the myocardium (Figure 2).

At rest, there were no differences in β between the two groups ($P = 0.33$); however, Group 1 had significantly reduced rBV and MBF compared to Group 2 ($P < 0.001$). After adenosine stress, both groups had significant increases in rBV, β, and MBF relative to their resting values ($P < 0.001$). However, after adenosine stress, Group 1 had significantly lower rBV, β, and MBF values compared to Group 2 ($P < 0.001$). β_{reserve} and MBFR were significantly lower in Group 1 when compared to Group 2 ($P < 0.001$) (Table 2).

HgbA1c > 7.1 group has higher proportion of abnormal segments and lower proportion of normal segments than HgbA1c ≤ 7.1 group, $P < 0.02$ (Figure 3).

When patients were stratified according to status of CAD diagnosis (known versus suspected), there was a significant inverse correlation between MBFR and HgbA1c% ($r = -0.279$, $P = 0.01$) in patients with suspected, but not known CAD. However, in those with known CAD, this inverse correlation was present, but not significant ($r = -0.117$, $P = 0.129$).

Using a MBFR cutoff value of >2 as normal, the variables of sex, age, obesity (BMI ≥ 30 kg/m^2), smoking status, dyslipidemia, duration of T2DM, presence of CAD, and/or hypertension were included in the univariate models, and HgbA1c% > 7.1% significantly increased the risk for having abnormal MBFR (unadjusted odds ratio: 1.84, 95% CI: 1.10–3.11, $P = 0.02$). After further adjustment for variables with likelihood test P value < 0.2 in univariate analysis, abnormal MBFR remained significant (adjusted odds ratio: 1.92, 95% CI: 1.12–3.35, $P = 0.02$) (Table 3).

3.3. Intra- and Interobserver Variability. For interobserver and variability of quantitative MCE perfusion analysis in our echo lab, the mean differences ± SE and the r for rBV reserve, β reserve, and MBF reserve were 5.85 ± 0.81 ($r = 0.371$, $P < 0.001$), 1.34 ± 0.04 ($r = 0.623$, $P < 0.001$), and 5.96 ± 0.17 ($r = 0.544$, $P < 0.001$), respectively, while for interobserver variability they were, 4.26 ± 0.61 ($r = 0.308$, $P < 0.001$), 0.57 ± 0.05 ($r = 0.528$, $P < 0.001$), and 1.72 ± 0.06 ($r = 0.528$, $P < 0.001$), respectively.

4. Discussion

Impaired coronary flow reserve (CFR) in T2DM patients has been demonstrated in studies using invasive coronary Doppler flow wires stress [15], and noninvasive, but ionizing radioactive nuclear techniques single-photon emission tomography (SPECT) [16]. We have previously shown that T2DM is associated with myocardial microvascular abnormalities as evidenced by abnormal myocardial perfusion determined by quantitative RTMPE. We compared the accuracy of the RTMPE determinations with SPECT and found

Table 1: Clinical characteristics of the study population ($N = 24$).

Characteristics	Group 1 ($n = 9$) (HgbA1c > 7.1%)	Group 2 ($n = 15$) (HgbA1c ≤ 7.1%)	P value[a]
Age (years)	68.14 ± 3.91	64.34 ± 3.02	0.34
Males	7 (77)	9 (60)	0.36
BMI	31.38 ± 2.43	34.78 ± 1.88	0.15
Current smoking	5 (56)	5 (33)	0.28
Hypertension	7 (78)	13 (87)	0.58
Duration of known DM (years)	9.60 ± 2.52	8.0 ± 1.56	0.74
Known CAD	8 (89)	9 (60)	0.11
Dyslipidemia[b]	8 (89)	15 (100)	0.38
Previous MI	4 (44)	6 (40)	0.83
Previous CABG	5 (56)	6 (40)	0.46
Previous PCI	2 (22)	6 (40)	0.36
Medications			
Statins	8 (89)	12 (80)	0.56
ACE inhibitors	4 (44)	8 (53)	0.67
Aspirin	5 (56)	10 (67)	0.59
Beta Blocker	7 (78)	10 (67)	0.56
Calcium channels blockers	2 (22)	4 (27)	0.81
Nitrates	2 (22)	3 (20)	0.89
Rest			
HR (beats/min)	65 ± 7	70 ± 14	0.77
SBP (mmHg)	146 ± 17	135 ± 23	0.21
DBP (mmHg)	78 ± 9	69 ± 11	0.08
RPP (beats/min × mmHg)	9426 ± 951	9388 ± 2382	0.88
Adenosine stress			
HR (beats/min)	74 ± 12	80 ± 14	0.33
SBP (mmHg)	143 ± 8	124 ± 17	0.08
DBP (mmHg)	72 ± 12	65 ± 15	0.26
RPP (beats/min × mmHg)	10600 ± 1951	10086 ± 2142	0.44

BMI: body mass index; MI: myocardial infarct; CABG: coronary artery bypass graft; PCI: percutaneous coronary intervention; ACE: angiotensin converting enzyme; HR: heart rate; SBP: systolic blood pressure; DBP: diastolic blood pressure; RPP: rate-pressure product. Continuous variables were presented as mean ± standard deviation. Categorical variables were presented as numbers and percentages (%). [a]Chi-square test for categorical data and independent t-test for continuous data comparison. [b]Dyslipidemia was defined as total cholesterol (>210 mg/dl, or LDL > 130 mg/dl, or HDL < 35 mg/dl) or receiving lipid lowering medication.

that a CFR cutoff of 1.9 provided sensitivity of 79%, specificity of 63%, and accuracy of 66% in T2DM patients [12].

In the current study, we sought to explore if there was a relationship between adequacy of T2DM control and abnormality of myocardial perfusion, using the noninvasive quantitative technique of RTMPE in patients with known or suspected CAD and referred for stress testing. We found that patients with poor control of their T2DM, defined as HgbA1c > 7.1%, had poorer myocardial microcirculatory perfusion, as evidenced by lower MBFR values. We also noted that this relationship was stronger in those patients that did not have established (known) CAD, but rather in those with known CAD.

Our findings are in alignment with the conclusions of the United Kingdom Prospective Diabetes Study (UKPDS) [17] which demonstrated that effective control of hyperglycemia can significantly reduce diabetic microvascular disease, especially when control is achieved early in the course of the diabetes. Similarly, a meta-analysis showed that lowering HgbA1c in T2DM decreased the risk of CHD and all-cause mortality. No specific thresholds were identified above which patients were at greater risk of developing CAD, but the greatest risk reduction was found in those with HgbA1c level below 7% [18]. However, there were still concerns regarding confounding factors affecting myocardial blood flow in T2DM subjects, such as age, gender, coexisting CAD, lipid disorders, smoking, and hypertension [19, 20]. In our study, after adjusting for those possible confounders, HgbA1c > 7.0% remained significantly associated with abnormal MBFR (defined as <2). We concluded that HgbA1c > 7.0% is an independent risk factor for having lower MBFR in T2DM patients.

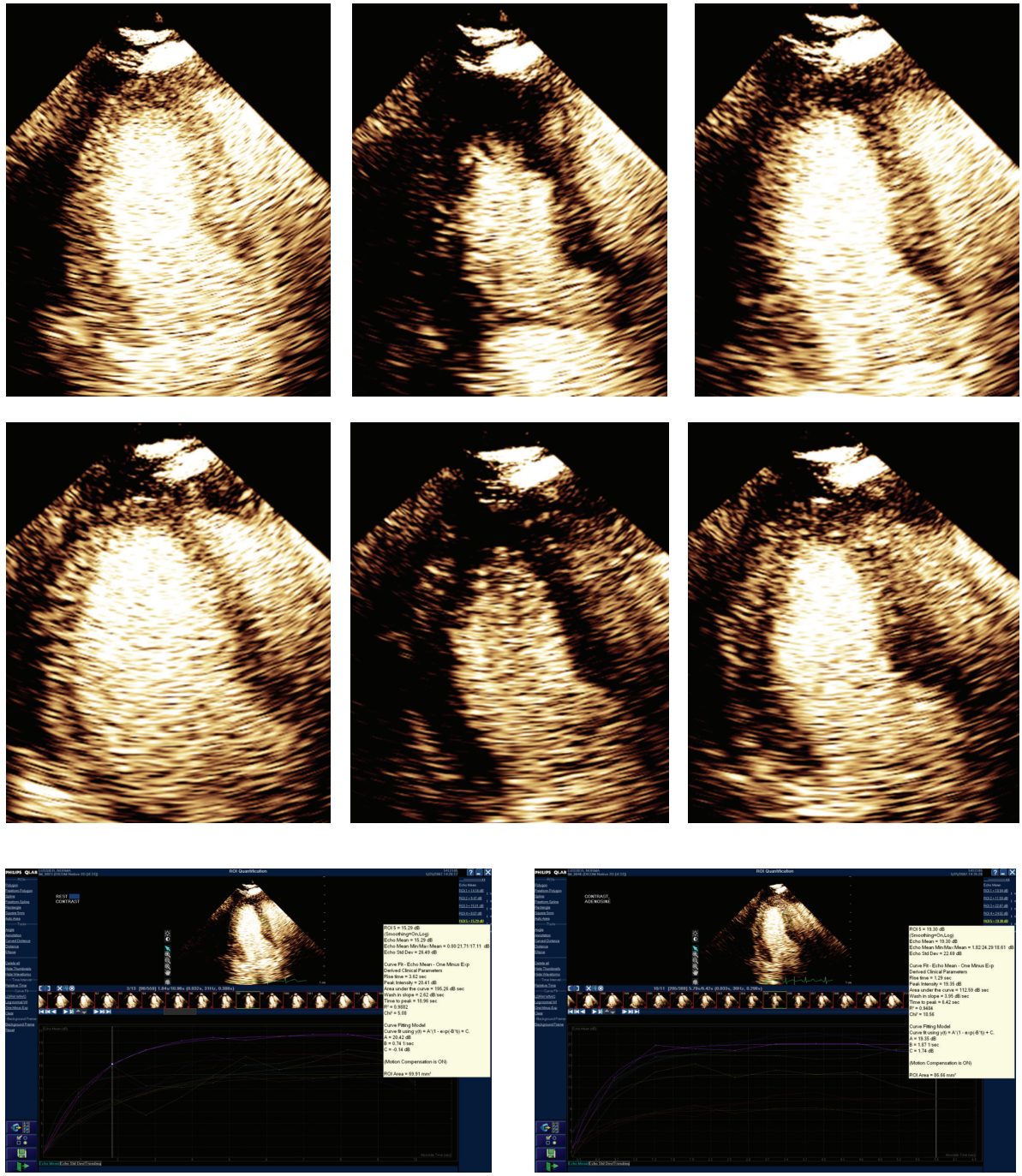

Figure 2: An example of T2DM patient perfusion sequence and analysis curve of 6 segments. 2D imaging of predepletion; depletion (flash), repletion (from left to right; upper: baseline; below: stress), and analysis curve of 6 segments (upper: baseline, below: stress).

Conversely, the ACCORD and VADT trials [21, 22] suggested that intensive diabetic control was disadvantageous in patients with long-standing diabetes and established CAD. Our results would support the findings in those studies as well, in that we observed that HgbA1c level had less apparent effect on the MBFR in those with established CAD, suggesting that when the microcirculation has been irreversibly affected by chronic glycemic elevations, there may be no benefit to achievement of tighter glycemic control.

Diabetes is a major risk factor for heart failure (HF) due to associated microvascular dysfunction. Recent studies [23, 24] have demonstrated that HgbA1c levels can predict HF hospitalization in T2DM patients, after adjusting for baseline cardiac and renal function. Moreover, HgbA1c has

Table 2: Comparison of quantitative myocardial perfusion parameters by segmental analysis.

	Group 1 (n = 107) (HgbA1c > 7.1%)	Group 2 (n = 165) (HgbA1c ≤ 7.1%)	P^g (rank sum test, 2- tail P value)
rBV_{rest}	0.20 (0.15, 0.29)[a]	0.28 (0.21, 0.34)[d]	<0.001
rBV_{stress}	0.28 (0.17, 0.38)	0.41 (0.30, 0.47)	<0.001
$rBV_{reserve}$	1.39 (0.91, 1.78)	1.41 (1.08, 1.81)	0.51
β_{rest}	5.12 (3.60, 9.52)[b]	6.16 (4.00, 8.92)[e]	0.33
β_{stress}	4.51 (2.56, 18.46)	9.91 (3.91, 20.26)	0.004
$\beta_{reserve}$	1.38 (0.71, 1.84)	1.87 (0.98, 2.49)	<0.001
MBF_{rest}	1.05 (0.52, 1.91)[c]	1.62 (0.91, 2.57)[f]	0.002
MBF_{stress}	1.20 (0.51, 4.80)	3.08 (1.31, 7.93)	<0.001
MBFR	1.71 (0.74, 3.22)	2.41 (1.31, 4.05)	0.004

rBV: rest relative blood volume; β: myocardial blood flow velocity; MBF: absolute myocardial blood flow; MBFR: myocardial blood flow reserve. Data is presented as median (25% IQR, 75% IQR). [a,b,c]Wilcoxon sign rank test for comparison between baseline and stress perfusion parameters (rBV, β, and MBF) in HgbA1c > 7.1% group, $P < 0.001$. [d,e,f]Wilcoxon sign rank test for comparison between baseline and stress perfusion parameters (rBV, β, and MBF) in HgbA1c ≤ 7.1% group, $P < 0.001$. [g]Wilcoxon rank sum test for comparison between HgbA1c > 7.1% group and HgbA1c ≤ 7.1% group.

Table 3: Univariate and Multivariate risk factors for abnormal MBFR < 2.

Variable	Odds ratio	95% confidence interval	P value	Odds ratio	95% confidence interval	P value
Sex	1.25	0.75, 2.06	0.39	—		
Age	1.02	1.00, 1.04	0.08	—		
Obesity BMI	1.13	0.66, 1.90	0.66	—		
Smoking status	1.54	0.95, 2.54	0.08	1.83	1.09, 3.12	0.02
Dyslipidemia	1.05	0.41, 2.73	0.92	—		
Duration of DM	1.12	0.69, 1.85	0.64	—		
Known CAD	1.21	0.74, 2.00	0.44	—		
HTN	1.0	0.98, 1.01	0.65	—		
High HgbA1c%	1.84	1.10, 3.11	0.02	1.92	1.12, 3.35	0.02

Obesity BMI defined as BMI > 30 and high HgbA1c% defined as >7.1%. Variables with likelihood test P value < 0.2 in univariate analysis were included in the multivariate logistic regression model and provided final adjusted odds ratios and 95% confidence intervals (95% CI).

been shown to be an independent risk factor for HF regardless of the presence of coronary risk factors or development of coronary heart disease during follow-up. Thus, our results using RTMPE suggest the unique potential for serial utilization of a relatively inexpensive, portable, and noninvasive tool to assess microvascular function in T2DM receiving therapy targeted to HgbA1C level in order to prevent cardiac dysfunction.

Our study is limited primarily by the small numbers in our sample size. However, the findings are in accordance with observations from large clinical trials and suggest a potential mechanistic explanation for the dichotomous clinical interpretations of these trials. Our study suggests that further research using noninvasive techniques such as RTPME along with HgbA1c assessments may be useful to assess cardiovascular risk prediction in the growing T2DM population.

We also did not have concurrent angiographic evidence for the presence or absence of epicardial disease and relied on the history to establish a diagnosis of epicardial CAD. However, when we separated the patients into two groups by degree of glycemic control based upon HgbA1c levels, we did not find any significant differences in clinical characteristics or prior history of CAD.

At the current time, RTMPE is an off-label technique. However, it can be readily performed by using the same equipment and following the similar technical recommendations as currently approved for the on-label indication of left ventricular opacification and endocardial border enhancement during the assessment of left ventricular function.

5. Conclusions

Our findings are consistent with the published evidence that optimal glycemic control results in a lower incidence of abnormal microvascular perfusion. The observed inverse relationship between HgbA1c and MBFR in T2DM patients without known CAD suggests that improved glycemic control may reduce the likelihood of subsequent cardiovascular events. HgbA1c > 7.0% is an independent risk factor for having abnormal MBFR in T2DM patients. Early detection of and therapeutic intervention for vascular complications in T2DM patients are important to decrease the risk of CAD

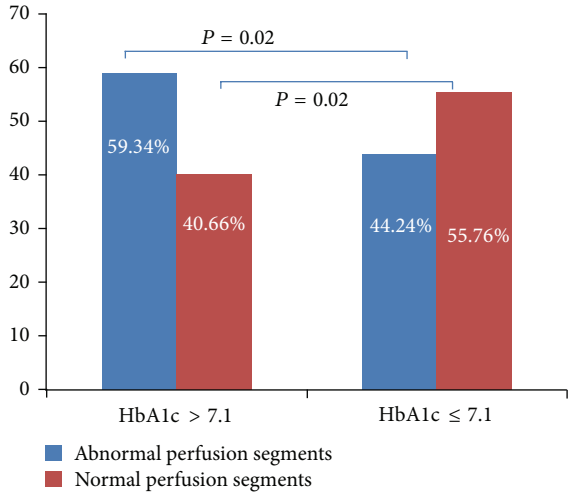

FIGURE 3: Percent of abnormal and normal perfusion segments compared between two groups.

events. RTMPE provides a noninvasive, readily available opportunity for early detection of microvascular complications in T2DM patients, prior to development of clinical symptoms. In addition, the quantitative measurement of RTMPE used to assess microvascular function may be useful for future monitoring of therapeutic interventions in T2DM patients.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Decreased Neuronal Bursting and Phase Synchrony in the Hippocampus of Streptozotocin Diabetic Rats

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Diabetic encephalopathy is one of the complications of diabetes. Cognitive dysfunction is the main consequence. Previous findings from neuroanatomical and *in vitro* electrophysiological studies showed that the structure and function of the hippocampus is impaired in diabetes, which may underlie the cognitive dysfunction induced by diabetes. However the study of electrophysiological abnormality of hippocampal neurons in intact networks is sparse. In the current study, we recorded the spontaneous firing of neurons in hippocampal CA1 area in anesthetized streptozotozin (STZ)-diabetic and age-matched control rats. Profound reduction in burst activity was found in diabetic rats. Compared to control rats, the intra-burst inter-spike intervals were prolonged significantly in diabetic rats, while the burst ratio and the mean number of spikes within a burst decreased significantly. Treatment with APP 17-mer peptide retarded the effects of diabetes on these parameters. In addition, the average PLV of diabetic rats was lower than that of control rats. These findings provide *in vivo* electrophysiological evidence for the impairment of hippocampal function in STZ-diabetic rats, and may have some implications in the mechanisms associated with cognitive deficits in diabetes.

1. Introduction

Due to ageing, high calorie diet, and physical inactivity, the prevalence of diabetes mellitus (DM) appears to be rapidly increasing. The term DM describes a metabolic disorder of multiple aetiologies characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both [1]. It causes a series of complications including vascular disorder, retinopathy, nephropathy, and peripheral neuropathy which may be disabling or even life-threatening. Currently, the idea that diabetes mellitus has negative impacts on the central nervous system has been widely accepted based on a substantial body of studies [2–7]. Moderate cognitive impairment has been observed in both human beings and animal models with type I or type II diabetes mellitus [5, 8–10]. Recently, diabetes mellitus has attracted considerable attention not only because of its negative effect on the brain but also because of its association with other neurodegenerative diseases [11–14]. Evidence showed that the incidence of Alzheimer's disease (AD) was higher in individuals with diabetes than those without [14, 15].

Streptozotocin- (STZ-) induced rat model is a model of type 1 diabetes mellitus which has been used extensively in studies of the pathophysiology of diabetes [6]. STZ rats show end-organ damage affecting eyes, kidneys, blood vessels, and nervous system. Spatial learning impairment related to the damage of central nervous system has been reported in STZ rats [16, 17]. Although the mechanism underlying cognitive impairment in diabetes is still unclear, accumulating evidence shows that functional or anatomical change of hippocampus is one of those possible mechanisms [18]. As we know, the hippocampus is a critical structure involved in learning and memory in the brain [19]. Several lines of research

have studied the effects of experimental diabetes on the synaptic plasticity in the hippocampus. Neuroanatomical research showed that the dendritic morphology of hippocampal neurons was altered in STZ-diabetic rats, including the decrease in the dendritic length and the density of dendritic spines of pyramidal cells [10]. Brain glutamate receptor abnormality was also found in hippocampus of STZ rats [20, 21]. Moreover, the cell proliferation decreased dramatically in the dentate gyrus of STZ-induced diabetic rats [22]. It has been demonstrated that the minor alteration in synaptic efficacy happened earlier than the anatomical abnormality in neurodegenerative disorders [23, 24]. Electrophysiological methods can provide the possibility to detect the alteration in synaptic function earlier, and it will be more valuable in the assessment of the efficacy of therapy. Previous *in vitro* electrophysiological studies have shown that the expression of long-term potentiation (LTP) in hippocampal slices was impaired in diabetic rats, whereas long-term depression (LTD) was enhanced [16, 25]. However, little is known about the *in vivo* electrophysiological changes of hippocampal neurons in diabetes mellitus.

Amyloid precursor protein (APP) is a transmembrane protein expressed in many tissues and concentrated in the synapses of neurons, which plays important roles in the regulation of several important cellular functions, especially in the nervous system, where it is involved in synaptogenesis and synaptic plasticity [26]. APP has six isoforms in central nervous system (CNS), of which APP-695 is the most important [27]. Amyloid precursor protein 17-mer peptide (APP 17-mer peptide) is an active fragment (319–335) of APP-695 in the nervous system that mediates various neuronal activities and functions. It has been reported that APP 17-mer peptide is an effective therapy for diabetes-induced impairment of cognition [28, 29]. APP 17-mer peptide improved the spatial learning and memory when tested by Morris water maze and it increased the synaptic density of diabetic rats. The effect of APP 17-mer peptide on diabetic encephalopathy may be exerted by regulating the metabolism of A β [30]. In the present study, the efficacy of APP 17-mer peptide was evaluated by observing its effect on the electrophysiological changes in diabetic encephalopathy.

Here we recorded the spontaneous firing of neurons in area CA1 in STZ-induced diabetic rats and age-matched control rats by *in vivo* extracellular recording, aimed to explore the effects of diabetes on the function of hippocampus. In addition, the efficacy of APP 17-mer peptide was evaluated in this paper. The neuronal firing pattern in the hippocampus was changed in STZ-diabetic rats and APP 17-mer peptide partially reversed the effect of diabetes mellitus.

2. Experimental Procedures

2.1. Animals. Adult male Sprague-Dawley rats (starting weight ~250 g) were used in this experiment. Animals were housed in a temperature- and humidity-controlled environment and given food and water *ad libitum*. Diabetes mellitus was induced by intraperitoneal injection of streptozotocin (60 mg kg^{-1}) (Sigma, St. Louis, MO) dissolved in 0.1 mol/L sodium citrate buffer (PH 4.4). Control rats were injected with saline only. The animal was fasted for 12 hours prior to receiving the injection. Nonfasting blood glucose concentrations of STZ-treated rats and age-matched control rats were measured three days after STZ injection. In all STZ-injected rats, animals with blood glucose level in blood samples obtained by tail prick >15 mmol/L were declared diabetic and selected. All procedures described in this study were reviewed and approved by the Local Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Experimental Design. Diabetic animals selected into the experiment were divided into 2 groups randomly. One group received subcutaneous injection of APP 17-mer peptide (0.34 μg, daily) in the back of the neck (APP group, $N = 6$). The other group received vehicles alone (DM group, $N = 6$). Another six rats served as age-matched control group (Ctrl group, $N = 6$). The body weights and blood glucose concentrations were measured every two weeks. The changes of body weight and blood glucose level are shown in Figure 1. After 12 weeks, the *in vivo* extracellular recording was performed.

2.3. Surgery and Extracellular Recording. The rats were anesthetized with 20% urethane (1.0 g kg^{-1} i.p.) initially. Supplemental doses (1/3 original dose) were given as needed to maintain appropriate level of anesthesia. The depth of anesthesia was monitored by heart rate, respiratory rate, toe pinch reflex, and eye blink reflex. After being anesthetized, the animal was placed in a stereotaxic apparatus. The scalp was removed, and a small bone window (2 × 2 mm) was drilled above the hippocampus (centered at AP: –3.6 mm and ML: +0.22 mm from Bregma, based on the atlas of rat brain [31]) for *in vivo* extracellular recording. Multiunit recording was obtained with 16-channel (2 × 8) polyimide-insulated tungsten microwire arrays (Tucker-Davis Technologies Inc, Alachua, FL, wire diameter 33 μm, electrode spacing 175 μm). After the dura was removed, electrodes then were slowly lowered stereotaxically into CA1 area (~3 mm ventral to dura) by a FHC hydraulic microdrive (FHC, Bowdoinham, ME) until action potentials with pyramidal cell firing characteristics were recorded [32]. A stainless screw placed in the contralateral hemisphere served as ground electrode. The spontaneous firing of neurons (30 min) was bandpass filtered (300–3000 Hz), amplified (RA16PA Medusa PreAmps, TDT), and digitized at 25 kHz with TDT multichannel acquisition system (RX5-2, Tucker-Davis Technologies, Gainesville, FL). For some neurons, wide-band pass (0.5–3000 Hz) signals were recorded and local field potentials were obtained by filtering the raw data with a pass band of 0.5–100 Hz. All the recordings were performed during 1 to 1.5 hours after anaesthesia and in a relatively consistent depth of anaesthesia. The data were then stored in the hard disc for offline sorting and analysis.

2.4. Data Analysis. The spike sorting was performed by principal component analysis in Opensorter software (Tucker-Davis Technologies; Gainesville, FL) and putative single units

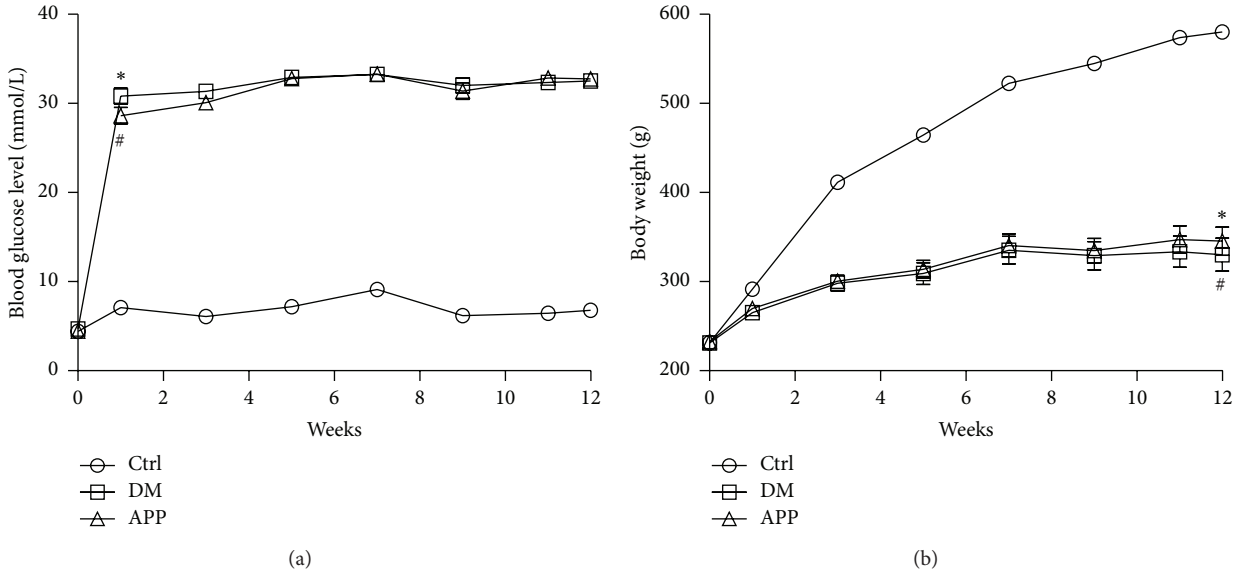

Figure 1: The blood glucose levels and body weights of the rats in Ctrl ($N = 6$), DM ($N = 6$) and APP ($N = 6$) groups. *Significant difference between diabetic rats (both in DM and APP group) and control rats ($P < 0.05$).

were isolated by semiautomatic clustering. Neurons with signal-to-noise ratio >3 : 1 were selected to perform further analysis. Autocorrelograms and other sequential analyses were calculated using custom software (developed using MATLAB, Mathworks, MA). Putative pyramidal cells and interneurons were distinguished by the spike duration [33], discharge frequency [34], and autocorrelation function [35]. Those neurons with spike duration >0.5 ms, background firing rate <10 Hz, and a peak at 3–5 ms in autocorrelogram were classified as pyramidal neurons. Putative pyramidal cells were classified into bursting cells and nonbursting cells. Complex spike bursting is characteristic of pyramidal cells in the intact hippocampus. It has been demonstrated that compared to single spikes, bursts have a special role in synaptic plasticity [36]. To explore the changes of synaptic plasticity in diabetes, we mainly analyzed the burst firing pattern in this study, based on the hypothesis that impairment of synaptic plasticity in diabetes should have representation in changes of burst features. A burst is identified by the criteria that it consists of 2–6 successive spikes with short interspike intervals (≤6 ms) and decreasing amplitude [32]. To reduce the error rate in spike sorting when two or more bursting neurons were recorded from one channel at the same time, we abandoned those channels which had two or more bursting neurons difficult to be cleanly sorted (Figure 2(a), upper panel). Further analysis including burst ratio (defined as bursts per 500 spikes), mean number of spikes in bursts, intraburst interspike intervals, and spike amplitude attenuation within bursts were performed then. For intraburst interspike interval, only those neurons with number of spikes within burst >3 were used for analysis, and only peak-peak intervals between the first and second spikes (ISI1) and the second and the third spikes (ISI2) were calculated. Spike amplitude attenuation within a burst was determined by dividing the amplitude of the second spike (or third spike) in the burst by the amplitude of the first spike. To make sure that a burst is coming from a single neuron, we strictly inspected the burst selected for further analysis. For a putative bursting neuron, first, we visually selected 20 bursts with very similar shapes as standard bursts. Then, we calculated the mean ± SD of ISIs and attenuation of amplitudes of those 20 bursts and automatically selected those bursts whose intraburst ISIs and the attenuation of amplitudes are very close to standard bursts (≤ mean ± 2 SD) by a program for further analysis.

To explore the changes of temporal coordination in diabetic rats, the degree of phase-locking between neuronal spike firing and LFP delta oscillation was calculated. The delta oscillation was obtained by band-pass filtering the hippocampal LFP into band frequency 1–4 Hz, and the filtered delta band was decomposed into phase $\Phi(t)$ component by using the Hilbert transform [37]. The phase value of a spike was determined by the instantaneous phase value of delta wave at the time when the spike occurred. For further quantifying the degree of spikes' phase locking to hippocampal delta rhythm, we used the phase-locking value (PLV) which is defined as [38]

$$\mathrm{PLV} = \left\| \frac{1}{N} \sum_{i=1}^{N} e^{j\phi_i} \right\|, \tag{1}$$

where ϕ_i is the phase value of the ith spike, while N is the number of spikes. PLV was compared between control and diabetic rats. Meanwhile, the spike train of individual unit was broken up at the troughs of the hippocampal delta rhythm and the resulting segments were stacked up to form the delta-triggered rasters (Figure 6(b), upper panel).

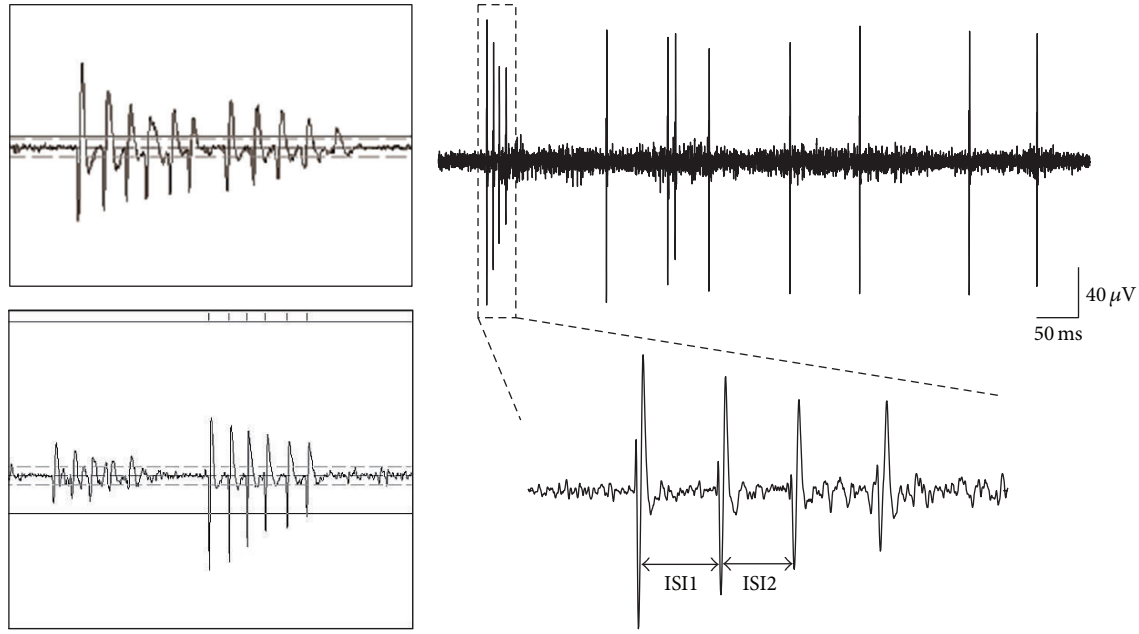

Figure 2: Examples of spontaneous firing of pyramidal neurons recorded from hippocampal CA1 area. (a) Examples of two bursting neurons recorded in one channel. The upper panel showed two bursting neurons whose spike waveforms were too close to be cleanly sorted. The lower panel was an example that the burst can be cleanly sorted to a single unit. (b) An epoch of spontaneous firing which includes a four-spike burst and several single spikes to show the quality of our signal. The burst is exaggerated below. ISI1 is the interval between the second and the first spike while ISI2 is the interval between the third and the second spike.

The lower panels of Figure 6(b) were the corresponding phase value distributions.

2.5. Statistical Analysis. Data was expressed as means ± standard error of mean (SEM). One-way repeated measures analysis of variance (ANOVA) and Tukey's post hoc test were used to analyze blood glucose level, body weight, and the burst ISI data. Statistical comparison of burst ratio and number of spikes within bursts was made by applying a one-way Kruskal-Wallis test and post hoc analysis with the Mann-Whitney test. Difference in the number of bursting and nonbursting pyramidal cells among the three groups was calculated by chi-square method. If there was statistical significance, further analysis was made to compare the difference between groups by partition of chi-square test. Student's t-test was performed to compare the phase-locking values between control and DM groups. $P < 0.05$ was considered statistically significant.

2.6. Histology. After completion of the electrophysiological recording, lesion was made at all the tips of all electrodes by passing direct current (10 μA, 10 s). Then the rats were deeply anesthetized and perfused intracardially with 100 mL of 0.9% saline solution followed by 300 mL of 4% buffered formalin phosphate solution. The brains were sectioned coronally at 40 μm thickness. The sections were mounted on slides and stained with hematoxylin and eosin for localizing the electrode tip.

3. Results

3.1. Blood Glucose Levels and Body Weight. There were no differences in body weight and blood glucose levels before the administration of STZ among the three groups (Figure 1). However, three days after STZ administration, the blood glucose levels of diabetic rats (30.8 ± 1.6 mmol/L for DM group and 28.6 ± 1.6 mmol/L for APP group) were significantly higher than that of the control rats (7.1 ± 0.3 mmol/L, $P < 0.0001$) and maintained a higher level until the start of the electrophysiological recording. The blood glucose levels of control rats fluctuated within a narrow range during the observation period. As expected, body weight of the control rats increased steadily during the observation period while that of the animals in the other two groups increased slowly. Twelve weeks after the STZ administration, body weights of rats in DM (334 ± 31 g) and APP groups (347 ± 26 g) were significantly lower than that of control rats (574 ± 9 g, $P < 0.0001$). There were no significant differences in body weight and blood glucose levels between rats in DM and APP group. The data of blood glucose levels and body weight reported in this paper are consistent with previous reports [10, 39].

3.2. Changes of Burst Firing Pattern. Data from electrodes falling outside hippocampal CA1 area were excluded from dataset. A total of 210 neurons were recorded from area CA1. The numbers of different types of neurons in control, DM, and APP group are shown in Table 1. The detected bursting

Table 1: Number of different types of cells recorded from CA1 in the three groups.

Group	Pyramidal cell		Interneurons	Total
	Bursting	Nonbursting		
Ctrl	59	12	6	77
DM	26	38*	3	67
APP	35	23#$	8	66

Chi-square test showed that there was significant difference ($\chi^2 = 29.101$, $P < 0.001$) among groups. Further partitioning chi-square analysis showed that there was also significant difference between groups (*DM versus Ctrl, $\chi^2 = 26.039$, $P < 0.001$; #APP versus Ctrl, $\chi^2 = 8.36$, $P < 0.01$; $APP versus DM, $\chi^2 = 4.733$, $P < 0.05$).

neurons in the control group were significantly more than in the other two groups.

An epoch of spike trains including isolated spikes and bursts recorded from CA1 area is shown in Figure 2. For bursting neurons, ISI1 and ISI2 were compared among groups. As shown in Figure 3, ISI1 of rats in DM group (4.58 ± 0.1 ms; $n = 26$) was significantly longer than that in control group (3.72 ± 0.05 ms; $n = 59$; $P < 0.001$) and APP group (4.15 ± 0.15 ms; $n = 35$; $P < 0.01$). There was also significant difference between ISI1 of control group and that of APP group ($P < 0.05$). For ISI2, the same difference was also observed between DM group (5.02 ± 0.09 ms; $n = 26$) and control group (3.94 ± 0.07 ms; $n = 59$; $P < 0.001$) as well as DM group and APP group (4.32 ± 0.17 ms; $n = 35$; $P < 0.001$). ISI2 of APP group was slightly longer than that of control group ($P < 0.05$). In addition, the comparison between ISI1 and ISI2 among the three groups showed that ISI2 was significantly longer than ISI1 in all the three groups (Figure 3).

To describe the burst rate during the recording period, we used the burst ratio as one index of burst propensity, which was calculated by the number of bursts per 500 spikes. The mean burst ratio of control group (47.7 ± 4.0; $n = 59$) was significantly higher than that of DM group (30.1 ± 4.1; $n = 26$; $P < 0.05$) and APP group (33.9 ± 3.8; $n = 35$; $P < 0.05$) (Figure 4(a)). A significant difference was also found in burst ratios between DM group and APP group ($P < 0.05$). Our results suggested that APP 17-mer peptide partially reversed the decreased burst ratio in diabetic rats.

In addition, we also analyzed the difference in number of spikes within bursts between groups (Figure 4(b)). In general, the mean of spike counts of control rats (4.6 ± 0.3; $n = 59$) was a little larger than that of DM group (3.5 ± 0.3; $n = 26$; $P < 0.05$). Treatment of APP 17-mer peptide had an effect on the decreased number of spikes within bursts observed in diabetic rats. APP 17-mer peptide increased the reduced spike number to 3.9 ± 0.1 ($n = 35$; $P < 0.05$) that was observed in diabetic rats to some degree but a difference from control rats was still observed ($P < 0.05$).

Decreasing amplitude is a prominent characteristic of burst firing. Analysis of the spike amplitude attenuation within bursts showed that, for the 2nd spike, there was a decrease in amplitude attenuation in diabetic rats (0.81 ± 0.01) compared with control rats (0.78 ± 0.02, $P < 0.05$, Figure 5).

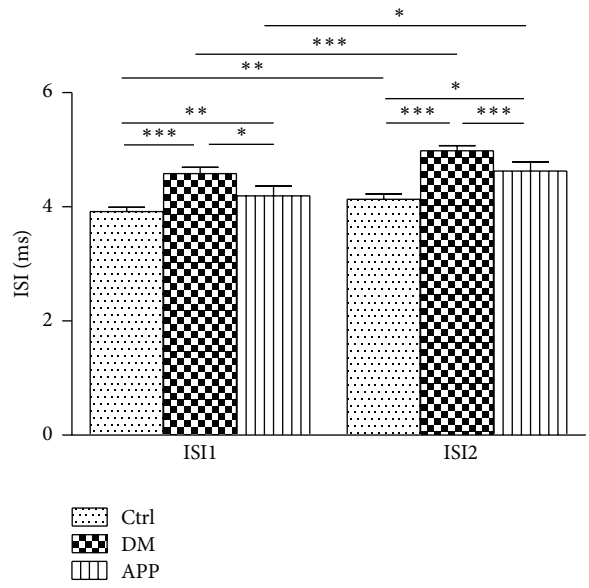

Figure 3: The intra- and intergroup comparison of intraburst ISI1 and ISI2. ISI1 and ISI2 of neurons ($n = 59$) recorded from DM rats were much longer than that of control rats ($n = 26$). APP 17 peptide treatment significantly decreased neuronal intraburst ISI1 and ISI2 ($n = 35$) compared with DM rats, while significant differences were also found between rats in control and APP group. In addition, ISI2 was a little longer than ISI1 in all the three groups ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, Mann-Whitney test). "n" represents number of neurons.

The decrease of amplitude attenuation was more apparent in the third spikes (0.59 ± 0.02 for Ctrl group; 0.68 ± 0.02 for DM group, $P < 0.05$). For the third spike, treatment of APP 17-mer peptide partially reversed the decrease of amplitude attenuation found in diabetic rats (0.64 ± 0.02 for APP group, $P < 0.05$).

3.3. Impaired Temporal Coordination Measured by Phase Locking Value. To investigate the change of temporal coordination in diabetic brains, phase-locking analysis between spike and local field potential (LFP) was performed. Delta waves were broadly observed in the recorded LFPs in all rats of DM and control groups. Analysis of the power of LFP showed that low-frequency, high-amplitude waves peaking between 1–4 Hz are the dominant activity pattern, which have been reported often during deep slow wave sleep and anesthesia [40, 41]. A typical example of phase-locking phenomenon was shown in Figure 6(a). The raw trace was filtered into delta oscillations (1–4 Hz) and spikes. Spikes were phase-locked to the trough of the delta waves. Figure 6(b) shows the phase value raster (upper panel) and corresponding phase value distribution (lower panel) of two typical neurons in control and DM rats. The difference in the degree of phase locking between control and DM group is shown in Figure 6(c). There was significant difference between PLV of control rats (0.54 ± 0.03) and that of DM rats (0.25 ± 0.03, $P < 0.001$). Our results showed that the phase-locking between spikes and delta oscillations in DM rats was poorer than that in control

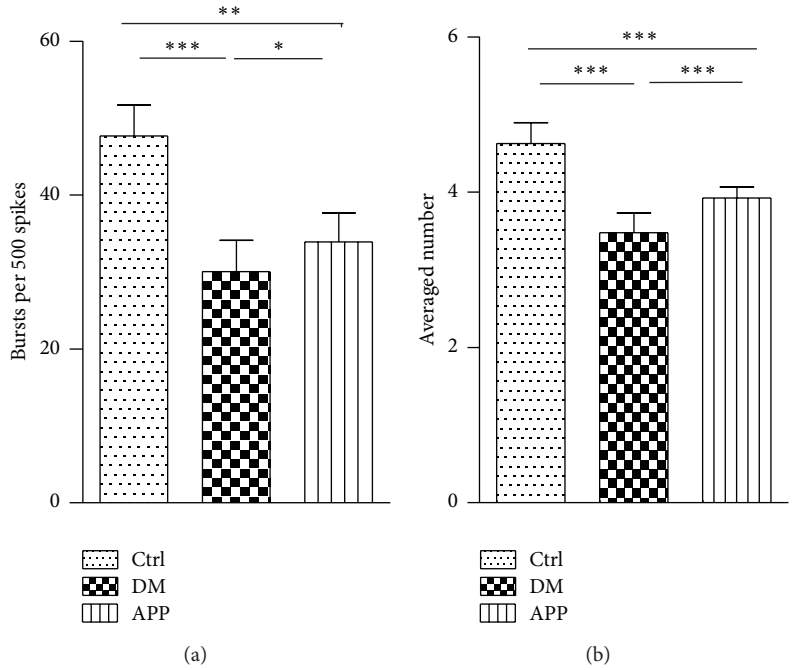

FIGURE 4: Comparison of burst ratio and number of spikes within burst between groups. The burst ratio of neurons from diabetic rats ($n = 26$) is significantly lower than that of control rats ($n = 59$, $P < 0.001$, Mann-Whitney test). Decrease of the number of spikes within bursts in diabetic rats was also observed ($P < 0.001$, Mann-Whitney test). Compared to that of diabetic rats, APP 17-mer peptide increased the burst ratio and number of spikes within bursts ($n = 35$, $P < 0.05$ for burst ratio; $P < 0.001$ for number of spikes within bursts, Mann-Whitney test) but still had significant difference with control rats ($P < 0.01$, Mann-Whitney test, for burst ratio; $P < 0.001$ for number of spikes within bursts). "n" represents number of neurons.

rats. To investigate if the network oscillation of DM rats is generally different from that of control rats, we compared the power of LFP activity between control and DM groups. Delta power ratio (delta power ratio was calculated by ratio of power in LFP frequency band 1–4 Hz to that in the whole LFP band (0.5–100 Hz)) was compared between neurons recorded from control and DM groups (Figure 6(d)). Our data showed that, compared to that of Ctrl group (0.75 ± 0.12, $N = 6$), delta power ratio decreased significantly in DM group (0.62 ± 0.08, $N = 6$, $P < 0.05$).

4. Discussion

In the present work, we used *in vivo* multichannel electrophysiological method to study the effect of diabetes mellitus on the spontaneous activity of hippocampal neurons in STZ-diabetic rats. Our results demonstrate that burst activities of hippocampal pyramidal neurons were impaired in STZ-diabetic rats, including decreased number of bursting neurons, prolonged intraburst interspike intervals, decreased burst ratio, and the number of spikes within a burst. APP 17-mer peptide can partially reverse the observed negative effect. Meanwhile, the phase synchrony between control and diabetic rats was also analyzed. Phase-locking value between single neuronal spiking and delta oscillation (1–4 Hz) of local field potential was significantly decreased in diabetic rats. The changes observed in this work may be correlated with the impairment of cognitive function in STZ-induced diabetes.

The dramatic reduction in burst activity defines possible mechanisms that may underlie the cognitive impairment observed in diabetes. Specifically, bursts as a unit of neural information have been postulated to play important roles in brain function [36, 42–44]. Compared with single spikes, presynaptic bursting of action potentials can be more reliably transmitted to the postsynaptic neurons and increase the probability of information transmission between neurons [45, 46]. Moreover, several lines of evidence indicated that bursts have a special role in synaptic plasticity [36]. Bursts may enhance synaptic plasticity at both presynaptic inputs that initiate a burst [36, 47] and postsynaptic potentiation caused by supralinear summation of excitatory postsynaptic potentials (EPSPs) at postsynaptic targets [48, 49]. It has been shown that the processes of synaptic modification seemed very sensitive to the types of bursts. The number of spikes within burst was related to the degree of synaptic modification. Bursts with two spikes produce little synaptic modification, and bursts with three spikes induce some LTP or LTD, while bursts with four spikes induce nearly maximal LTP or LTD [44]. Thus, the decreased number of spikes within burst in diabetic rats would be expected to decrease the synaptic efficiency.

A very interesting finding in the present study is that the intraburst interspike intervals in diabetic rats are significantly longer than those in control rats. It has been proposed that the fine temporal structure of burst has role in neural computation when bursts are analyzed as unitary events [50, 51].

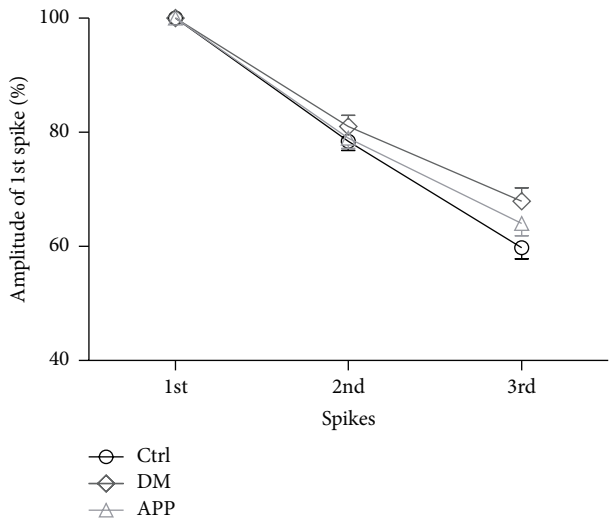

Figure 5: Comparison of intraburst amplitude attenuation between the three groups. Amplitude attenuation within bursts was reduced in diabetic rats ($n = 26$) either for the 2nd/1st spike ($P < 0.001$, Mann-Whitney test) or for the 3rd/1st spike ($P < 0.001$, Mann-Whitney test). Treatment of APP 17-mer peptide partially reversed the decrease in amplitude attenuation ($n = 35$, $P < 0.001$, Mann-Whitney test, for the second spike; $P < 0.001$ for the third spike). The difference in amplitude attenuation between APP and Ctrl group ($n = 59$) only was significant at the third spike ($P < 0.001$, Mann-Whitney test). For the second spike, no difference was observed ($P = 0.314$, Mann-Whitney test). "n" represents number of neurons.

Changes in the interspike interval may have pronounced influence on synaptic modification. Whether in the protocol of LTP elicited by high-frequency stimulation or primed burst potentiation elicited by burst stimulation, or short-term facilitation elicited by paired pulse stimulation, the interstimulus intervals have enormous effect on the post-synaptic responsive efficacy [52]. Previous studies observed the effects of complex spike trains in LTP/LTD induction by using paired bursts of spikes with varying pre/postintervals and burst frequency [53, 54]. Their results showed that in addition to pre/postinterval, synaptic modification was also found to depend on the firing frequency within each burst. The magnitude of LTP increases with the burst frequency. It is often assumed that the shorter the interspike interval within the burst, the better: the summed postsynaptic potential activated by presynaptic bursting of spikes is larger when the interval between the spikes is smaller [55].

Exactly how the STZ treatment came to reduce the burst activity is unknown but could involve alteration in presynaptic afferent fiber or changes in transmitter release. The decrease of NMDA receptor may be one of the possible causations. It is generally thought that bursting arises from activation of NMDA receptors and opening of L-type calcium channels. Thus, decrease in NMDA receptors will lead to a lack of sustained NMDA receptor-mediated membrane depolarization and then results in the decreased probability of successive action potential generation and the reduction of the number of closely spaced spikes and burst activity [56–58]. It has been shown that, after 12 weeks of STZ-diabetes, the level of the NR2B subunit of the NMDA receptor is decreased by 40%. Moreover, the phosphorylation of the NR2A/B subunits by Ca2+/calmodulin-dependent protein kinase II is reduced in STZ-diabetic rats [39]. Our results are in line with the *in vitro* electrophysiological findings that the expression of NMDA-dependent LTP in the CA1 is impaired in diabetes [5, 59]. Further investigation will be required to clarify mechanism underlying the reduction of burst activity in diabetes.

Oscillatory activity plays a crucial role in hippocampal functions that are mainly expressed during diverse behaviors and various stages of sleep. Delta oscillation appears as a large-amplitude slow frequency (1–4 Hz) extracellular rhythm of local field potentials during deep slow-wave sleep as well as under urethane anaesthesia, which is believed to be important in enhancing synaptic excitability in the hippocampus [60, 61]. In this paper, delta power ratio was found to be significantly decreased in diabetic rats. It is possible that the differences in bursting behavior observed in this paper may be due to the differences in network states between the two groups. More comprehensive experiment should be designed to testify this hypothesis in future study. Phase-locking analysis showed that PLV of diabetic rats was significantly lower than that of control rats, which demonstrated that STZ-induced diabetes impairs the temporal coordination between neuronal spiking and slow oscillation of population activity in the hippocampus. The possible explanation for the disruption of neural coordination is the loss of neurons [62] and dendritic spines [10] in diabetic brains leading to the alteration in the capacity of individual neuron receiving inputs from cell assemblies. Thus, the coordination between the firing of single neuron and the activity of cell assemblies is disrupted. Recently, Sigurdsson et al. proposed that the impaired hippocampal-prefrontal synchrony may be a fundamental component of the pathophysiology underlying schizophrenia [63]. Our study provided further evidence for the theory that abnormal neural synchrony may be one of the mechanisms underlying cognitive dysfunction related to brain disorders.

Application of polypeptide-based therapy against neurodegenerative diseases has been reported in the past [64–67]. It is a promising field of treatment for neurodegenerative diseases. Previous studies reported that amyloid precursor protein (APP) is a protein with neurotrophic function [28]. APP 319-335 segment (APP 17-mer peptide) of APP 695 was demonstrated to retard neuronal degeneration in diabetic mice by regulating the metabolism of Aβ [30]. At the behavioral level, when spatial learning and memory were tested in water maze, APP 17-infused diabetic rats showed increased memory retention [28]. In the present paper, the degree of firing pattern changes in diabetic rats with APP 17-mer peptide treatment was smaller than that in diabetic rats, which implied that treatment of APP 17-mer peptide can retard the degeneration induced by STZ diabetes.

In summary, our findings describe *in vivo* electrophysiological observations of hippocampal neuronal activity in STZ-diabetic rats. We found that neuronal burst activity was significantly reduced in diabetic rats. From the perspective

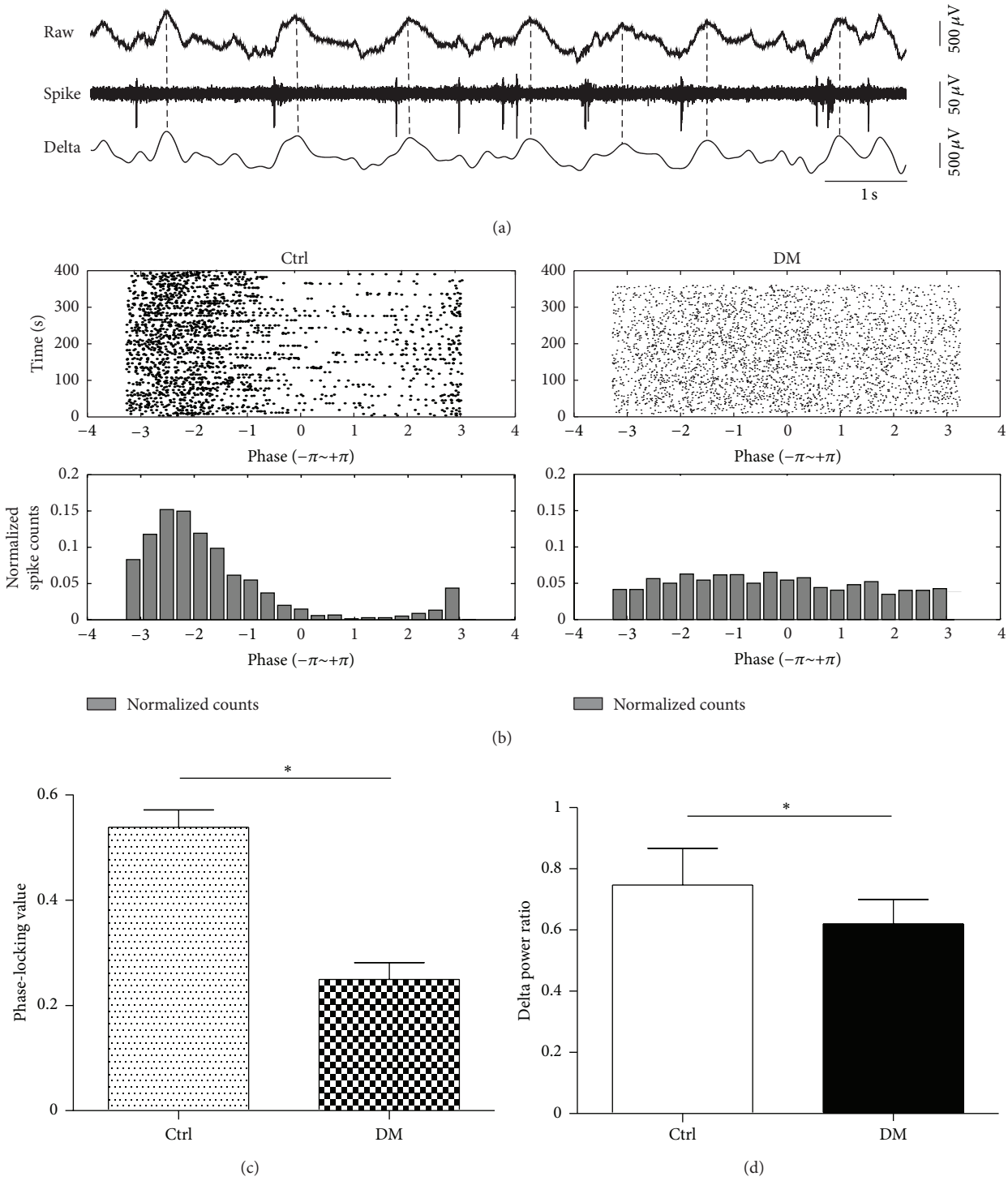

Figure 6: Phase-locking of hippocampal neurons. (a) Epoch of wide-band raw trace as well as corresponding delta oscillation of LFP (band-pass filtered in the frequency band of 1–4 Hz) and spike train. The phase-locking between spike and delta oscillation of LFP is apparent. (b) Phase value rasters (upper panel) and the corresponding phase value (lower panel) distributions of two typical neurons in the control and DM groups. Control rats showed stronger phase-locking phenomenon than diabetic rats. (c) Comparison of phase-locking values between diabetic and control rats. The PLV in control rats ($n = 59$) was significantly higher than that in diabetic rats ($n = 26$) ($P < 0.05$, Student's t-test). (d) Comparison of delta power ratio between Ctrl and DM groups. There was significant difference between Ctrl ($N = 6$) and DM ($N = 6$) groups ($P < 0.05$, Student's t-test). "N" represents number of rats and "n" represents number of neurons.

of local circuit, phase synchrony was also impaired by STZ treatment. These alterations may be responsible for the impairment of hippocampal function in STZ-induced diabetic rats. Our results at least partly imply the mechanisms underlying the cognitive deficits in diabetes.

Our study has limitations in the following two aspects. (1) The electrophysiological recording was performed on anesthetized rats. Because diabetic rats may have altered metabolism and blood brain barrier, the anesthetics may have different effect on control rats and diabetic rats. To minimize the different effect of anesthetics, the dose of anesthetics was reduced slightly (about 1/10) in diabetic rats. At the meantime, we visually inspected respiration rate and heart rate carefully and frequently during the experiment to make sure that the respiration and heart rate were maintained in a consistent level among the three groups. Even so, we cannot completely exclude the possibility that the results reported in this paper were partly due to the effect of anesthesia. However, our results showed that there was no difference in blood glucose levels and body weight between diabetic rats and diabetic rats with APP 17-mer peptide treatment, and the same dosage of urethane was delivered to the two groups, while the changes of firing pattern in the two groups were different significantly. Our results strongly implied that the changes of firing pattern were the consequence of STZ-induced diabetes. The conclusion will be testified in the future work on awake rats. (2) The microelectrode used in this study was multielectrode arrays rather than tetrode. Due to the dense distribution of neurons in hippocampus, it is hard to cleanly sort signals recorded from one single electrode into single units. However, considering that our data has high SNR and we abandoned that the neurons cannot be cleanly sorted, we believe that the error in sorting was controlled in an acceptable range.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Zhimei Qiao and Kangning Xie contributed equally to this work and should be considered co-first authors. Zhimei Qiao and Guoliang Li are co-corresponding authors of this work.

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Review Article

Pathogenesis of Chronic Hyperglycemia: From Reductive Stress to Oxidative Stress

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Chronic overnutrition creates chronic hyperglycemia that can gradually induce insulin resistance and insulin secretion impairment. These disorders, if not intervened, will eventually be followed by appearance of frank diabetes. The mechanisms of this chronic pathogenic process are complex but have been suggested to involve production of reactive oxygen species (ROS) and oxidative stress. In this review, I highlight evidence that reductive stress imposed by overflux of NADH through the mitochondrial electron transport chain is the source of oxidative stress, which is based on establishments that more NADH recycling by mitochondrial complex I leads to more electron leakage and thus more ROS production. The elevated levels of both NADH and ROS can inhibit and inactivate glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively, resulting in blockage of the glycolytic pathway and accumulation of glycerol 3-phospate and its prior metabolites along the pathway. This accumulation then initiates all those alternative glucose metabolic pathways such as the polyol pathway and the advanced glycation pathways that otherwise are minor and insignificant under euglycemic conditions. Importantly, all these alternative pathways lead to ROS production, thus aggravating cellular oxidative stress. Therefore, reductive stress followed by oxidative stress comprises a major mechanism of hyperglycemia-induced metabolic syndrome.

1. Introduction

Type 2 diabetes is generally an overnutritional disease [1–3]. It is caused by insulin resistance and insulin secretion impairment induced gradually and mainly by high blood glucose in conjunction with other factors such as obesity, aging, genetic predisposition, and physical inactivity [4–9]. Persistent overnutrition creates a steady level of high blood glucose that is toxic to macrovascular and microvascular systems [10–12], an effect known as glucotoxicity [13–17]. While oxidative stress is thought to contribute to the pathogenesis of glucotoxicity during the development of diabetes and diabetic complications [18–26], reductive stress due to excess NADH [27–33] generated by high blood glucose has attracted less attention. In this review, by following the mechanisms of NADH production and recycling, I highlight evidence that reductive stress followed by oxidative stress comprises the fundamental pathogenic mechanisms of chronic hyperglycemia in the development of diabetes and diabetic complications.

2. Euglycemia

A normal level of blood glucose below 100 mg/dL is tightly maintained, regulated, and achieved by rate of glucose uptake by all tissues and rate of glucose synthesis by the liver [34] and to a less magnitude by the kidney [35]. Approximately, 75% of the body's total glucose is consumed by insulin-insensitive tissues including the brain, red blood cells, the liver, and the gut, while the rest is consumed by insulin-sensitive tissues including muscle [36]. Postprandially, a rapid increase in blood glucose content stimulates insulin secretion, resulting in a temporary increase in blood insulin concentration known as hyperinsulinemia. The increases in blood concentrations of both glucose and insulin coordinately inhibit glucose production by the liver and facilitate glucose uptake by insulin-insensitive tissues [37]. Therefore, euglycemia is strictly maintained, which is highly dependent not only on proper insulin secretion from the β-cells upon nutritional stimulation but also on insulin action in the liver and peripheral tissues [37].

3. NADH and Reductive Stress

Electrons from aerobic breakdown of glucose are mainly stored in NADH for oxygen reduction and ATP production. Therefore, NADH is a reducing compound and an excessive amount of it can cause reductive stress [30, 32, 38–40]. Overproduction of NADH or lack of NAD^+ can induce the accumulation of NADH, leading to imbalance between NADH and NAD^+ and creating a condition known as pseudohypoxia [29, 41–44]. This is a condition under which oxygen cannot be effectively consumed. This would cause metabolic stress or metabolic syndrome as it often occurs in diabetes [44–47]. It should be noted that GSH and NADPH accumulation, tightly linked to NADH metabolism [48], can also induce reductive stress [39, 49–54]. As mitochondrial complex I is the major enzyme responsible for NADH recycling, impairment of complex I function can thus induce NADH accumulation and reductive stress [55] that could be linked to inhibition of insulin release by β-cells [56, 57].

4. Hyperglycemia, Elevated Levels of NADH, and Mitochondrial Electron Pressure

The glycolytic pathway breaks down nearly 80%–90% of the body's glucose, while the pentose phosphate pathway consumes the remaining 10%–20% under physiological condition [58, 59]. Under hyperglycemic condition, more glucose will flux through the glycolytic pathway that produces more pyruvate and acetyl-CoA, leading to more NADH production. As NADH is an electron carrier, excess amount of it will cause an electron pressure on the mitochondrial electron transport chain [40, 60–62]. This is particularly true for hepatocytes and pancreatic β-cells in that glucokinase (hexokinase D) is a supply-driven enzyme [63], and this enzyme is not inhibited by glucose-6-phosphate (G6P) [64–66]. Therefore, the more glucose the more G6P produced that will be broken down through glycolysis and Krebs cycle, leading to more NADH production. Figure 1 shows the major conventional pathways that can generate more NADH when glucokinase is used to phosphorylate glucose for glucose breakdown in tissues such as pancreas and liver [67–70].

5. NADH-Imposed Electron Pressure and Mitochondrial Superoxide Production

The electron pressure induced by overproduced NADH will place a heavy burden on mitochondrial complex I that is the major site for NADH recycling (Figure 2). Under this condition, complex I will respond within its capacity to oxidize more NADH to NAD^+, in an attempt to ameliorate the pseudohypoxic condition. An inherent nature of NADH flux through complex I is that more superoxide will also be made when more NADH is oxidized by complex I as this complex is also involved in proton pumping [71–73], leading to a proportional increase in electron leakage that will partially reduce oxygen to yield superoxide [71, 74–77]. This scenario could get worse under pseudohypoxic conditions as less NAD^+ is available for transporting electrons to oxygen [55], leaving more oxygen available for partial reduction by the leaked electrons from complex I and complex III, the latter being also involved in proton pumping [78–80]. It should be noted that complex II and dihydrolipoamide dehydrogenase could also produce superoxide [81–83].

6. Superoxide and Oxidative Stress

Superoxide is the precursor of all reactive oxygen species that at elevated levels can cause oxidative stress [84, 85]. As has been established, superoxide can be converted to hydrogen peroxide by superoxide dismutase; hydrogen peroxide can then be converted to form hydroxyl radical by metal ions [84, 86, 87]. In the meantime, superoxide can also react with nitric oxide to produce peroxynitrite ($ONOO^-$) [88, 89]. All these reactive species can cause oxidation of proteins, lipids, and DNA [90]. Consequently, an oxidative stress condition has fully developed due to a high level of NADH, achieving the transition from reductive stress to oxidative stress [43, 91–93]. Therefore, reductive stress is not the reverse of oxidative stress; it actually leads to oxidative stress [94, 95].

7. Inhibition of Glyceraldehyde 3-Phosphate Dehydrogenase and Alternative Glucose Metabolic Pathways

As has been discussed above, an oversupply of NADH can lead to overproduction of mitochondrial superoxide and other forms of ROS. These ROS can then impair the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [22, 96] that is very sensitive to oxidative modifications [21, 97–103] due to a redox-sensitive cysteine residue at its active center [104, 105]. Additionally, high level of NADH would also inhibit GAPDH activity [106]. Such impairments would collectively decrease the efficiency of glucose metabolism via glycolysis and Krebs cycle, inducing accumulation of glyceraldehyde 3-phosphate (G3P). Therefore, all the intermediate products above and including G3P will have to be disposed by pathways that branch off the glycolytic pathways (Figure 3) [107, 108].

8. The Branching-Off Pathways and Oxidative Stress

There have been five pathways [21] that can branch off the glycolytic pathway under chronic hyperglycemic conditions (Figure 3). These pathways are minor and insignificant in glucose metabolism under normoglycemic conditions, but can become major pathways to flux high level glucose. As will be discussed below, all the five pathways have been linked to ROS production, oxidative stress, and the pathogenesis of diabetes and diabetic complications [21, 109–115].

8.1. The Polyol Pathway. When blood glucose level is high, cellular metabolic pathways change, which usually leads to deleterious effects [5]. A major pathway that is activated in response to hyperglycemia is the polyol pathway [44, 116–118], in which glucose is reduced by aldose reductase to form

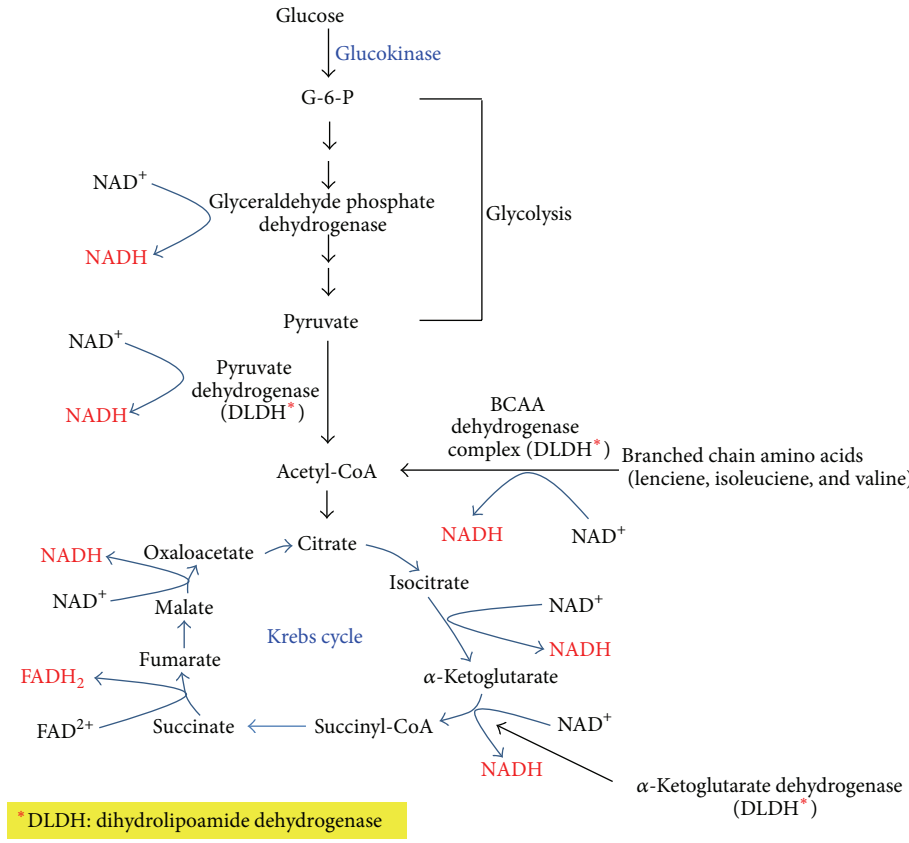

Figure 1: The conventional pathways that generate NADH by breaking down glucose via glycolysis and the Krebs cycle. The enzymes involved in NADH/NAD^+ recycling are shown. *DLDH stands for dihydrolipoamide dehydrogenase and is the component in each given enzyme complex that actually makes NADH from NAD^+ [191].

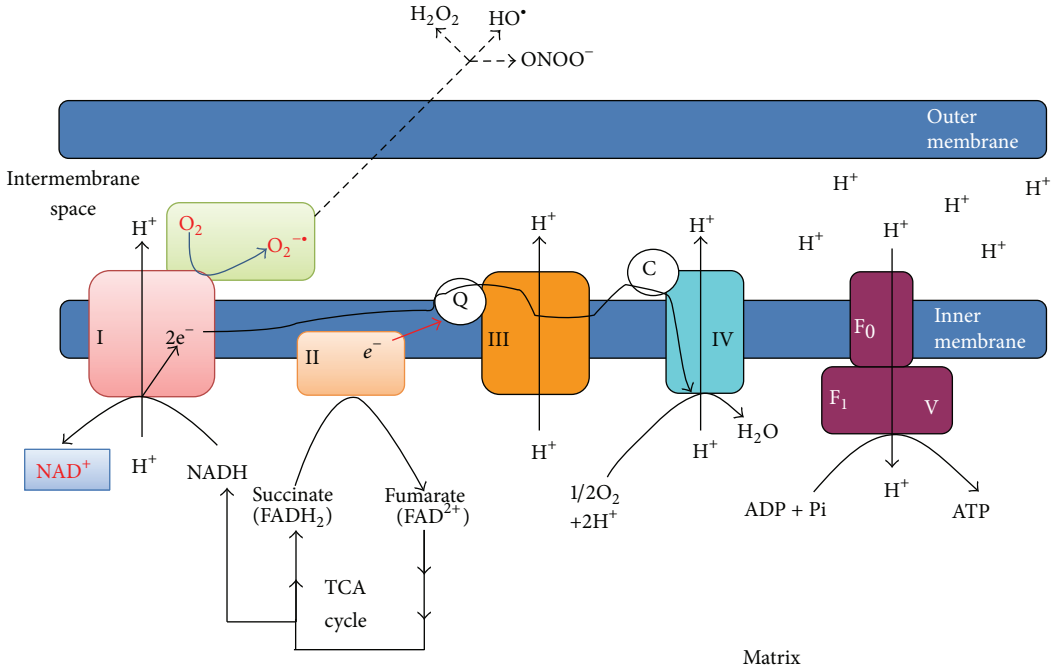

Figure 2: NADH oxidation by complex I in the electron transport chain. Electrons from NADH are transported via CoQ and cytochrome c to molecular oxygen. This process involves proton pumping that is tightly linked to superoxide production. ATP synthesis by complex V driven by the proton gradient is also shown.

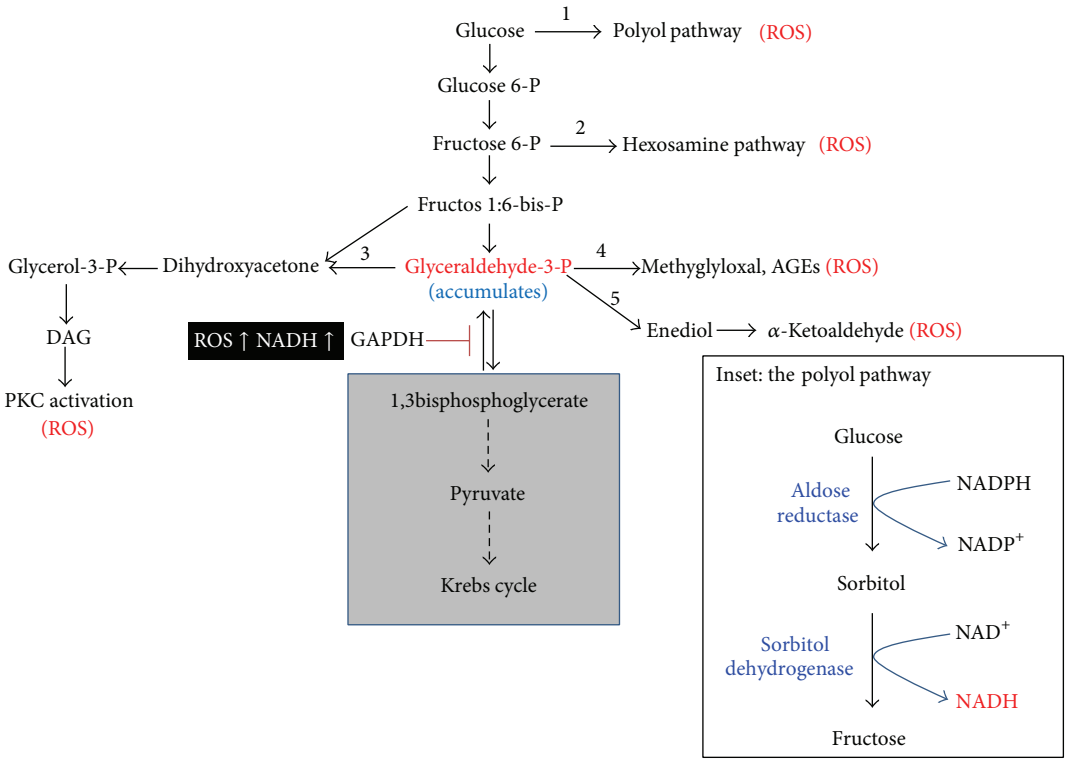

FIGURE 3: The branch-off pathways that are activated to dispose excess glucose when glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is inactivated by ROS. These five alternative pathways [21, 115], in addition to the electron transport chain shown in Figure 2, are linked to ROS production, thus further exacerbating oxidative stress. Inset shows the polyol pathway. Pathways in the grey area would no longer efficiently break down glucose when GAPDH is inactivated by posttranslational modifications.

sorbitol, and the formed sorbitol is then converted to fructose by sorbitol dehydrogenase. This pathway, as shown in Figure 3 (Inset), converts NADPH to NADH using two step reactions and leads to redox imbalance between NADH and NAD^+. As the ratio of NAD^+/NADH decreases due to an increase in NADH content, reductive stress can ensue. Because aldose reductase has a very high Km for glucose [119], it can only be activated by a high level of glucose. Hence, this enzyme could also be considered as a supply-driven enzyme [120, 121]. Under hyperglycemic conditions, the polyol pathway has been estimated to utilize more than 30% of the body's glucose [101]. Therefore, this pathway can also contribute significantly to reductive stress [32, 119] and has been thought to play an important role in the pathogenesis of diabetic complications [122–125].

Additionally, in the first reaction of the polyol pathway (Figure 3 inset), NADPH is consumed and, when NADPH level goes lower, so does reduced form of glutathione (GSH). This is because glutathione reductase needs NADPH to regenerate GSH from GSSG (oxidized form of glutathione) [126]. As GSH level goes lower, cellular antioxidant capacity can be compromised, resulting in elevated levels of reactive oxygen species that can attack macromolecules and induce oxidative damage [126]. Therefore, the polyol pathway is also a source of oxidative stress [127–129]. It should also be pointed out that activation of the polyol pathway in return will further decrease glucose consumption by the glycolytic pathway as sorbitol dehydrogenase competes with GAPDH for NAD^+ [130, 131]. Moreover, as nitric oxide synthase also uses NADPH as a cofactor, a lowered level of NADPH can lead to a decrease in nitric oxide production, thereby facilitating vasoconstriction and platelet aggregation [132].

8.2. The Hexosamine Pathway. This pathway branches off from fructose 6-phosphate in the glycolytic pathway. Fructose 6-phosphate is the substrate of the enzyme glutamine-fructose 6-P amidotransferase (GFAT), which is the rate-limiting enzyme for this pathway. GFAT makes glucosamine 6-P from fructose 6-P and the former is further converted to UDP-N-acetylglucosamine, which is the substrate for specific O-GlcNAc transferase that catalyzes posttranslational modifications of proteins via O-GlcNAc on serine and threonine residues [133–135]. Increased glucose flux through this pathway has been shown to be involved in ROS generation and oxidative stress [136–138] and has been implicated in diabetic complications [139–142].

8.3. The Protein Kinase C Activation Pathway. Fructose 1:6-bisphosphate can break down to form dihydroxyacetone phosphate and glyceraldehyde 3-phosphate with the former being readily isomerized to glyceraldehyde 3-phosphate under the action of triose phosphate isomerase. Accumulation of glyceraldehyde 3-phosphate can increase the synthesis of diacylglycerol that is an activator of protein kinase C

(PKC). PKC activation is known to be involved in elevating the content of TGF-β-1, endothelin-1, NF-κB, and vascular endothelial growth factor [22, 143, 144] and is also known to induce ROS production by NADPH oxidase that catalyzes one electron reduction of molecular oxygen to form superoxide [145–147]. Mechanistically, it has been established that PKC activates NADPH oxidase by phosphorylating the $p47^{phox}$ subunit, triggering the translocation of this subunit from cytosol to membrane whereby it assembles with other components to form an active NADPH oxidase that is capable of making superoxide from oxygen [148, 149]. PKC activation can also induce insulin resistance by inhibiting Akt-dependent nitric oxide synthase function [150].

8.4. Advanced Glycation End Products (AGEs). In addition to the polyol pathway, this pathway has also been thought to be a major mechanism of oxidative stress under hyperglycemic condition [151, 152]. High level of glucose can induce formation of methylglyoxal from glyceraldehyde 3-phosphate when GAPDH function is impaired. Methylglyoxal can modify proteins via glycation of amino groups on proteins [153, 154]. One of the major products is glycated hemoglobin (HbA1c) that has been used as a biomarker for diabetes [155, 156]. Therefore, this nonenzymatic process can greatly impair protein function. Moreover, this glycation pathway is known to liberate ROS [157, 158] and upregulate the expression of cell surface receptor for AGEs, leading to activation of the NF-κB signaling pathway and chronic inflammation [159–161].

8.5. The Glyceraldehyde Autoxidation Pathway. This pathway also branches off from glyceraldehyde 3-phosphate in the glycolytic pathway. Glyceraldehyde 3-phosphate is formed from fructose 1:6-bisphospate by the enzyme aldose. Under certain conditions, glyceraldehyde 3-phosphate can undergo autoxidation [162], a process that can generate hydrogen peroxide and α-ketoaldehydes in diabetes mellitus [21, 163].

9. Oxidative Stress, Diabetes, and Diabetic Complications

As discussed above, all the sources of ROS and oxidative stress can be traced back to high blood glucose and NADH overproduction. Therefore, chronic hyperglycemia would inevitably cause chronic reductive stress that leads to oxidative stress. As ROS production is a common feature of the above described pathways [119, 164], chronic oxidative stress certainly plays a central role in the development of diabetes and diabetic complications [22, 165, 166]. Indeed, it has been reported that ROS can induce insulin resistance [74, 167], impair insulin synthesis [168], and impair beta cell insulin secretion [97, 169]. Additionally, oxidative stress biomarkers have been shown to be increased in individuals who exhibit insulin resistance [170–173] or insulin secretion impairment [174–177], indicating a positive correlation between oxidative stress and insulin resistance and insulin secretion impairment. Moreover, numerous studies have also established that ROS are involved in the etiology of diabetic

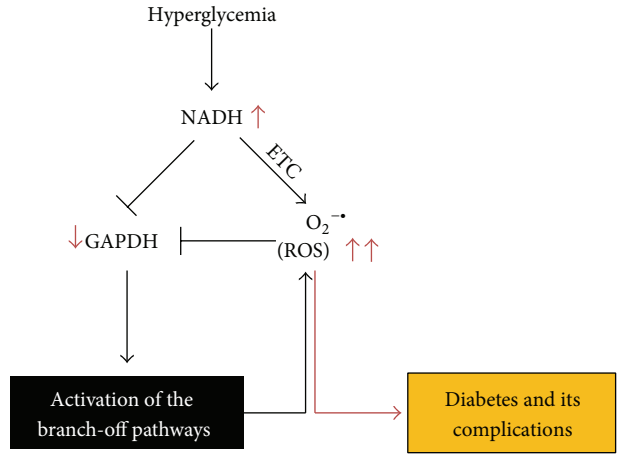

Figure 4: Hyperglycemia induces overproduction of NADH and mitochondrial ROS that inhibit GAPDH activity. This inhibition then activates the alternative glucose metabolic pathways, which further produce ROS involved in glucotoxicity that is responsible for the development of diabetes and diabetic complications. ETC: electron transport chain.

complications including retinopathy, neuropathy, cardiomyopathy, and nephropathy [123, 178–182]. Given that oxidative stress originates from NADH-imposed reductive stress [31, 183], attenuating hyperglycemia-triggered reductive stress may provide potential therapeutic approaches for preventing the development of diabetes and diabetic complications.

10. Conclusion

Persistent high blood glucose is highly toxic [16, 112]. It not only induces insulin resistance but also impairs insulin secretion by pancreatic β-cells [184]. Over time, hyperglycemia will produce detrimental effects on macrovascular and microvascular systems [185, 186]. Figure 4 summarizes schematically the pathways discussed in this review and their pathogenic roles in chronic hyperglycemia via NADH, ROS, and oxidative stress. As hyperglycemia results in excessive production of acetyl-CoA that feeds into the Krebs cycle, making excess NADH, mitochondrial electron transport chain is thus under heavy electron pressure [40, 60, 61]. Therefore, oxidation of the overproduced NADH by mitochondria will inevitably lead to production of more superoxide and hence more ROS [187, 188], which can in turn attack and inactivate GAPDH. This would trigger the accumulation of glycolytic metabolites upstream of glyceraldehyde 3-phosphate and activate the alternative glucose disposal pathways that all are linked to ROS production and hence increase the magnitude of oxidative stress [21, 189, 190]. Therefore, reductive stress followed by oxidative stress could serve as the major mechanism of glucotoxicity under chronic hyperglycemic conditions. An increase in NADH oxidation by mitochondria without an accompanying increase in ROS production may be a potential therapeutic approach for diabetes and diabetic complications.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research Article

Ethanol at Low Concentration Attenuates Diabetes Induced Lung Injury in Rats Model

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To observe the changes of lung injury when diabetic rats were treated with low concentration of ethanol (EtOH) and analyze the related mechanisms, male Sprague-Dawley (SD) rats were divided into control, diabetic (DM), and EtOH+DM groups. Diabetic rat was mimicked by injection of streptozotocin intraperitoneally. Fasting blood glucose (FBG) level, lung weight (LW), body weight (BW), and LW/BW were measured. The changes of lung tissue and Type II alveolar cell were detected. Pulmonary malondialdehyde (MDA) content and superoxide dismutase (SOD) activity were measured; meanwhile, ALDH2 mRNA and protein expressions were detected by RT-PCR and western blotting, respectively. Compared with control group, in DM group, SOD activity was decreased; FBG level, LW/BW, MDA content, ALDH2 mRNA, and protein expressions were decreased. Compared with DM group, in EtOH+DM group, SOD activity, ALDH2 mRNA, and protein expressions were increased; LW/BW and MDA content were decreased. The structures of lung tissue and lamellar bodies were collapsed in DM group; the injury was attenuated in EtOH+DM group. Our findings suggested that, in diabetic rat, pulmonary ALDH2 expression was decreased accompanying lung injury. EtOH at low concentration decreased diabetes induced lung injury through activating ALDH2 expression.

1. Introduction

According to an estimation of the World Health Organization (WHO), more than 366 million people worldwide will have diabetes mellitus in 2030; the urban population in developing countries is projected to double between 2000 and 2030. Diabetes mellitus (DM) is an endocrine and metabolic disease which affects almost all organs in our body. Although the lungs are not thought to be primarily affected by diabetes and lung physiological and structural abnormalities are happening in both type 1 and type 2 diabetes, the lung should be considered as a "target organ" [1].

Chronic hyperglycemia is considered as the main reason for diabetes complications. In the process of prolonged increased glucose concentration, reactive oxygen species (ROS) overproduction may be one of the key factors which induce organ injury. Decreased antioxidative function and increased oxidative stress were seen in the lung of diabetic rats and rabbits [2, 3]. Mitochondria dysfunction will cause oxidative stress injury which is one of the underlying factors for neurodegenerative diseases, diabetes, cardiovascular diseases, cancer, and so on [4]. Physiological hypoxia triggers the changes of mitochondrial redox and increases superoxide generation at Complex III in alveolar epithelial cells [5]. Mitochondrial aldehyde dehydrogenase 2 (ALDH2) is a nuclear-encoded mitochondrial enzyme that localizes in mitochondrial matrix [6]; ALDH2 protein had been detected in liver, lung, heart, kidney, testis, esophagus, stomach, colon, and pancreas [7]. Our previous study had reported that, in diabetic rats, ethanol (EtOH) at low concentration offered myocardial protection through activation of ALDH2 expression [8], and upregulation of ALDH2 also plays the protective effect in myocardial ischemia and reperfusion

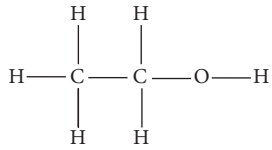


Figure 1: The biochemistry structure of ethanol.

injury, diabetes cardiomyopathy, and kidney and brain injury [9–12]; meanwhile, some researchers reported the association between ALDH2 and lung disease. Xu et al. reported that, in neonatal rat lung, after prolonged hyperoxic exposure, ALDH2 was downregulated. In lung epithelial cells, overexpression of ALDH2 attenuated hyperoxia-induced cell death through reduction of ROS, activation of ERK/MAPK, and PI3K-Akt signaling pathways [13]. But it remains unknown whether activation of ALDH2 expression can prevent diabetes induced lung injury. So in this study, we mimic diabetes model by intraperitoneal injection of streptozotocin (STZ) to observe the role of EtOH at low concentration in diabetes induced lung injury and analyze the related mechanisms.

2. Materials and Methods

2.1. Animals. Male Sprague-Dawley (SD) rats (200–250 g) obtained from the Animal Center of Bengbu Medical College were selected for the study. All animal studies were approved by the Animal Ethics Committee of Bengbu Medical College and performed in accordance with the ethical standards.

2.2. Chemicals and Reagents. Streptozotocin (STZ) was purchased from Sigma (St. Louis, MO, USA). Malondialdehyde (MDA) and superoxide dismutase (SOD) assay kits were from Nanjing Jiancheng Bioengineering Institute, China. Ethanol (EtOH, Figure 1) was purchased from Bengbu New Chemical Reagent Factory, China. The primers used were as follows: for ALDH2 forward: 5-GTG TTC GGA GAC 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′-GAT GGT GGG TAT GGG TCA GAA GGA C-3′ and reverse: 5′-GCT CAT TGC CGA TAG TGA TGA CT-3′ and the product size was 630 bp. Mouse anti-ALDH2 and anti-β-actin monoclonal antibodies were from Santa Cruz Biotechnology (CA). Goat anti-mouse secondary antibodies were purchased from Boston Co., Ltd., Wuhan, China.

2.3. Induction of Diabetes and Experimental Protocol. 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. The rats in control group were injected with a similar volume of sodium citrate buffer alone. The rats with plasma fasting blood glucose level higher than 16.7 mmol/L 72 h after injection were regarded as diabetic [8]. All rats were fed for eight weeks. Animals were randomly divided into control, diabetes (DM), and EtOH+DM groups, respectively ($n = 6$). In EtOH+DM group, DM rats were fed with 2.5% EtOH in their drinking water for one week to initiate drinking and then it was changed to 5% EtOH continuous access through the 8 weeks.

2.4. Detection of Fasting Blood Glucose (FBG) Level, Body Weight (BW), and Lung Weight (LW). The plasma FBG level and body weight (BW) were measured at the 8th week. Lung weight (LW) was determined after the rat was sacrificed. The ratio of LW/BW was calculated.

2.5. Detection of MDA Content and SOD Activity in Lung Tissue. At the end of the experimental period, 0.1 g lung tissue was homogenized in ice-cold PBS buffer. The supernatant was collected after centrifugation for 20 min (2000 rpm). The protein concentration was measured by the Bradford method. Malondialdehyde (MDA) content and superoxide dismutase (SOD) activity were detected according to the instruction manual.

2.6. Histological Observation by HE Staining. For histological analysis by light microscope, the lung tissue was harvested and fixed in 4% paraformaldehyde for 24 hours, embedded in paraffin and cut into 5 μm thick serial sections, and then stained with hematoxylin and eosin (HE) for light microscope observation. Lung injury degree was evaluated according to Mikawa's scoring standards: (1) alveolar congestion, (2) hemorrhage, (3) infiltration or aggregation of neutrophils in airspace or vessel wall, and (4) thickness of alveolar wall/hyaline membrane formation. Each item was scored on a 5-point scale as follows: 0: minimal damage, 1: mild damage, 2: moderate damage, 3: severe damage, and 4: maximal damage. The final lung injury score was the summation of the four items [14].

2.7. Ultrastructure Observation of Type II Alveolar Cell by Transmission Electron Microscope. Lung tissue was dissected and small pieces were fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer for 2 h and postfixed in 1% osmium tetroxide in 0.1 mol/L cacodylate buffer for 1 h. Ultrathin sections were cut and contrasted with uranyl acetate followed by lead citrate and observed with JEM-1230 transmission electron microscope (JEOL, Japan). The changes of Type II alveolar cell were observed.

2.8. Detection of Pulmonary ALDH2 mRNA by RT-PCR. The expression of pulmonary ALDH2 mRNA was detected by RT-PCR. Briefly, total RNA was extracted with TRIzol according to the manufacturer's instructions. A total RNA (2 mg) were reverse transcribed to cDNA, and PCR was performed by a routine method. PCR products were analyzed on 1% agarose gel. Quantification of the result was determined through measuring the optical density of the labeled bands; the value was normalized to β-actin intensity level.

2.9. Detection of Pulmonary ALDH2 Protein Expression by Western Blot. The pulmonary ALDH2 protein expression was detected by western blot [8]. Anti-ALDH2 (1 : 500) antibody was used. Mouse anti-β-actin antibody (1 : 500) was

Table 1: Changes of fasting blood glucose level (FBG), body weight (BW), lung weight (LW), lung weight/body weight (LW/BW), and lung injury score in different groups.

Group	FBG (mmol/L)	BW (g)	LW (g)	LW/BW (mg/g)	Lung injury score
Con	6.05 ± 1.02	443.53 ± 23.84	2.40 ± 0.43	5.39 ± 1.94	1.33 ± 0.81
DM	32.48 ± 3.20**	179.28 ± 28.46**	1.85 ± 0.38*	10.06 ± 1.79**	9.67 ± 1.21**
EtOH+DM	27.15 ± 6.67$^{**\#}$	243.75 ± 11.09$^{**\#\#}$	1.68 ± 0.20**	6.87 ± 0.56$^{\#\#}$	6.33 ± 0.82$^{**\#\#}$

$^{*}P < 0.05$ and $^{**}P < 0.01$ compared with Con; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with DM.

used as an internal control. The immunoblots were exposed to X-ray film and analyzed with a digital image system.

2.10. Statistical Analysis. All results were evaluated as mean ± S.E.M. Statistical comparisons were carried out by one-way variance analysis and the Newman-Keuls test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Changes of Fasting Blood Glucose Level. In contrast to control group, FBG levels in DM and EtOH+DM groups were increased significantly and FBG level in EtOH+DM group was lower than in DM group (Table 1).

3.2. Changes of Body Weight, Lung Weight, and the Ratio of Lung Weight to Body Weight. Compared with control animals, body weight (BW) and lung weight (HW) were significantly decreased in DM and EtOH+DM groups, and LW/BW was increased in DM group. In contrast to DM group, BW was increased and LW/BW was decreased in EtOH+DM group (Table 1).

3.3. Changes of SOD and MDA in Lung Tissues. In contrast to control rats, pulmonary SOD activity was decreased in DM group while MDA content was increased in DM and EtOH+DM groups. In EtOH+DM group, SOD activity was higher while MDA content was lower than in DM group (Table 2).

3.4. The Histological Changes of Lung Tissue. In control group, alveolar walls in lung tissue had unique shape and size, and hemorrhage and inflammatory infiltration were rare. In DM group, alveolar septum was thickened widely, infiltration of inflammatory cells and hemorrhage appeared, and the lung injury score was higher than in control group. The injury was ameliorated in EtOH+DM group than in DM group (Figure 2 and Table 1).

3.5. Ultrastructural Changes of Type II Alveolar Cell. In control group, many regular structure lamellar bodies appeared in Type II alveolar cell cytoplasm. In DM group, the structure of lamellar bodies was collapsed, discontinuous, and vacuolated. In EtOH+DM group, the injury degree was attenuated (Figure 3).

Table 2: Changes of SOD activity and MDA content in lung tissues in different groups.

Group	SOD activity (U/mg prot.)	MDA content (nmol/prot.)
Con	7.55 ± 1.94	3.93 ± 0.40
DM	4.07 ± 0.81**	5.63 ± 0.51**
EtOH+DM	6.45 ± 2.05$^{\#}$	4.67 ± 0.71$^{*\#\#}$

$^{*}P < 0.05$ and $^{**}P < 0.01$ compared with Con; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with DM.

Table 3: Changes of ALDH2 mRNA and protein expressions in lung tissues in different groups.

Group	ALDH2/β-actin mRNA	ALDH2/β-actin protein
Con	0.47 ± 0.05	1.17 ± 0.19
DM	0.35 ± 0.04**	0.85 ± 0.10**
EtOH+DM	0.42 ± 0.06$^{\#}$	1.14 ± 0.15$^{\#}$

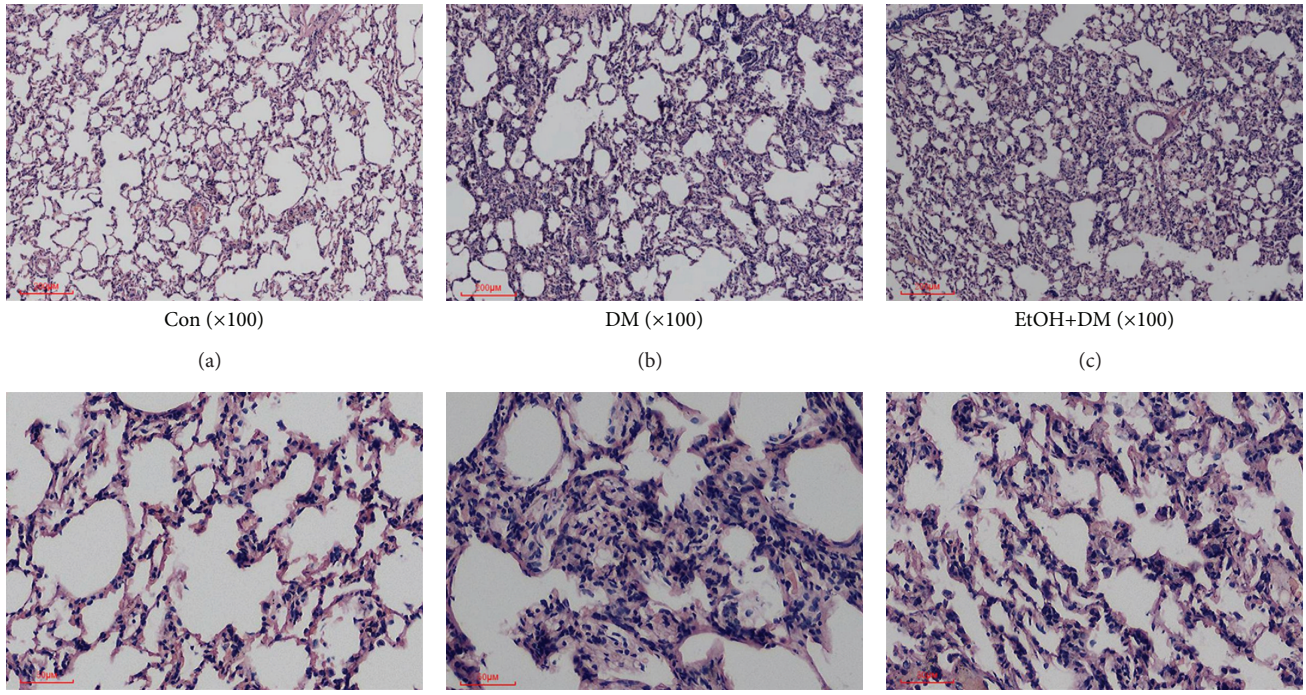
$^{**}P < 0.01$ compared with Con; $^{\#}P < 0.05$ compared with DM.

3.6. Changes of ALDH2 mRNA and Protein Levels in Lung Tissue. Compared with control group, the expressions of pulmonary ALDH2 mRNA and protein were decreased in DM group; compared with DM group, pulmonary ALDH2 mRNA and protein expressions were increased in ETOH+DM group (Figures 4 and 5 and Table 3).

4. Discussion

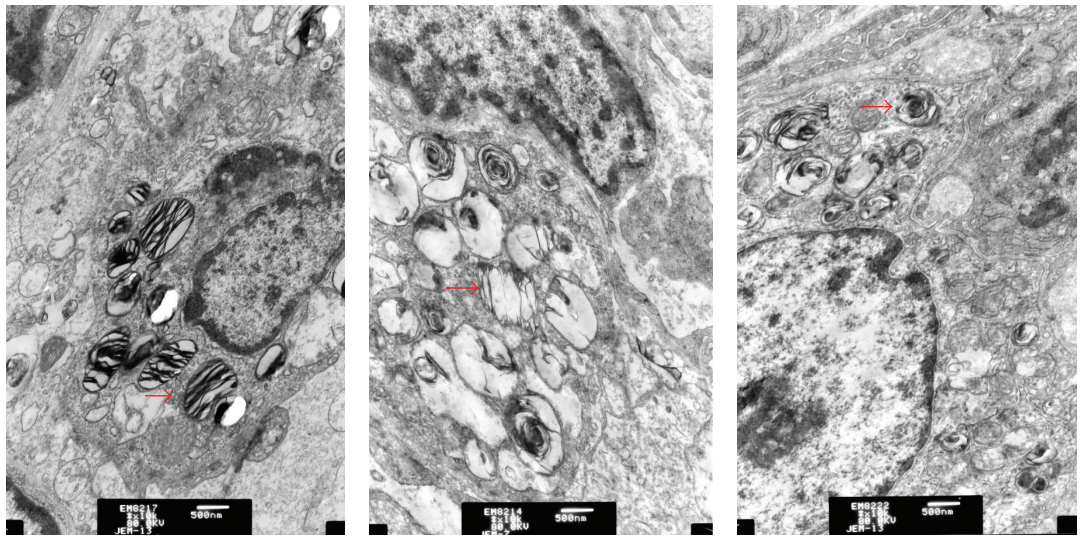
In the present study, we observed that lung oxidative stress injury occurred in diabetic rats, which was indicated by the increase of pulmonary MDA content and decrease of SOD activity; meanwhile, the decrease of pulmonary ALDH2 mRNA and protein expressions happened accompanying the happening of lung swelling, the destruction of pulmonary tissue, and Type II alveolar cell structure. When the diabetic rats were treated with EtOH at low concentration which was reported to activate ALDH2 expression [8, 10], lung ALDH2 mRNA and protein expressions were increased, lung oxidative stress injury and swelling degree were attenuated, the destruction of pulmonary tissue and Type II alveolar cell structure was alleviated, suggesting that downregulation of pulmonary ALDH2 was likely to be correlated with oxidative stress injury in diabetic rats, and activation of ALDH2 with EtOH at low concentration attenuated diabetes induced lung injury and oxidative stress overload.

In the development of diabetes, oxidative stress induced by chronic hyperglycemia plays a key role in the pathogenesis



Con (×100) (a)
DM (×100) (b)
EtOH+DM (×100) (c)
Con (×400) (d)
DM (×400) (e)
EtOH+DM (×400) (f)

Figure 2: Histological observation of lung tissue in different groups.



Con (a)
DM (b)
EtOH+DM (c)

Figure 3: Transmission electron microscopy pictures of Type II alveolar cell in different groups (×15 K).

of diabetes-related complications including lung diseases. Hyperglycemia can undergo autoxidation and generate oxygen radicals. Diabetes induces impairment of defense system, which is associated with reduced antioxidant capacity, abnormal activity, or expression of antioxidant enzymes [15]. An increase of MDA, a lipid peroxidation marker, accompanied by the depressing of SOD activity (one of the most important endogenous antioxidase enzymes), is assessed to imply oxidant damage. Oxidative stress can destroy the pulmonary structure. In our study, we observed that, in diabetic rat, pulmonary MDA content was increased with the decrease of SOD activity, and the structures of lung tissue and Type

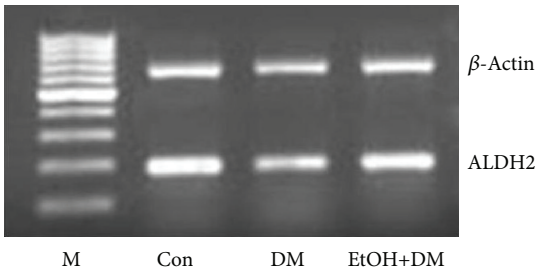


Figure 4: ALDH2 mRNA expression by RT-PCR.

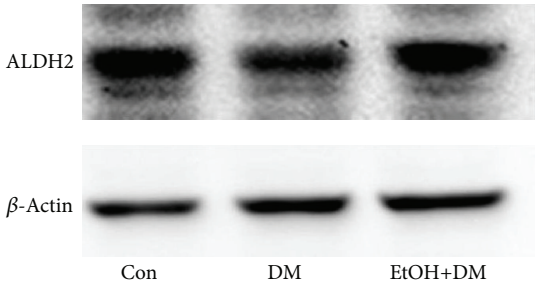


Figure 5: ALDH2 protein expression by western blot.

II alveolar cell were destroyed, suggesting the unbalance of lung antioxidative system induced lung injury in diabetes rats. When the diabetic rat was treated with EtOH at low concentration, pulmonary MDA content was decreased and SOD activity was increased accompanying the recovery of lung tissue and Type II alveolar cell structures in contrast to diabetic rat, suggesting that EtOH protected the lung tissue through antioxidative stress role.

ALDH2 is an enzyme that detoxifies aldehydes to carboxylic acids. The role of ALDH2 in oxidative stress had been reported by many researchers. Activation of ALDH2 with alda-1 alleviated cardiac ischemic and reperfusion injury with the decrease of 4-HNE in rodent models [8]. ALDH2 overexpression rescued neuronal survival against 4-HNE treatment in PC12 cells [16]. In male C57BL/6 mice, activation of ALDH2 with EtOH prevented renal ischemia and reperfusion injury with suppressed lipid peroxidation and increased SOD activity [12]. ALDH2 prevented ROS-induced vascular contraction in angiotensin-II induced hypertensive mice [17]. But few reports focus on the relationship of pulmonary ALDH2 and diabetes induced lung injury. ALDH2 overexpression attenuated hyperoxia-induced cell death in lung epithelial cells through reduction of ROS, activation of ERK/MAPK, and PI3K-Akt signaling pathways [13]. Since diabetes induced lung injury accompanied with oxidative stress, while increasing ALDH2 expression could decrease oxidative stress, so we investigated whether activating pulmonary ALDH2 expression by EtOH could decrease lung oxidative stress injury in diabetic rats. The results displayed pulmonary ALDH2 mRNA and protein expressions were decreased in diabetic rats, while EtOH treatment increased ALDH2 expression and, meanwhile, decreased oxidative stress injury; it suggested that, in diabetes induced lung injury, the aggravation of oxidative stress may be derived from the decrease of pulmonary ALDH2 expression and improving pulmonary ALDH2 expression could be against the happening of hyperglycemia induced oxidative stress.

It is worthwhile to note that, in our study, we selected EtOH to promote ALDH2 expression because there had been many papers reporting that EtOH at a suitable concentration could activate ALDH2 expression and play the protective effect [8, 10, 12, 18]. But in clinic, EtOH is difficult to apply for patients because of its toxicity and addiction. So selecting an appropriate drug to activate pulmonary ALDH2 expression may be beneficial for diabetic patients who had suffered from lung injury.

In conclusion, our results indicated that, in diabetes induced lung injury in rat model, pulmonary ALDH2 expression was decreased. Treatment with EtOH at low concentration can decrease diabetes induced lung injury through activating ALDH2 expression.

Conflict of Interests

The authors have no potential conflict of interests to declare.

Acknowledgments

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Research Article

Vitamin Status as a Determinant of Serum Homocysteine Concentration in Type 2 Diabetic Retinopathy

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We investigated the association of serum homocysteine levels and vitamin status with type 2 diabetic retinopathy. This study included 65 patients with and 75 patients without diabetic retinopathy. Patients with diabetic retinopathy had significantly higher serum homocysteine levels ($P < 0.001$), higher prevalence of hyperhomocysteinemia ($P < 0.001$), lower serum folic acid ($P < 0.001$), and vitamin B_{12} ($P = 0.014$) levels than those without diabetic retinopathy. Regression analysis revealed that homocysteine was an independent risk factor for diabetic retinopathy and there was a threshold in its serum level (13.7 μmol/L), above which the risk of diabetic retinopathy greatly increases (OR = 1.66, $P = 0.001$). Folic acid was associated with decreased odds for diabetic retinopathy (OR = 0.73, $P < 0.001$). There was a threshold in serum vitamin B_{12} level (248.4 pg/mL), below which serum homocysteine concentration significantly increases with decreasing serum vitamin B_{12} ($P = 0.003$). Our findings suggest that hyperhomocysteinemia is an independent risk factor for the development and progression of diabetic retinopathy. Decreased serum levels of folic acid and vitamin B_{12}, through raising serum homocysteine concentrations, may also affect the diabetic retinopathy risk.

1. Introduction

Progressive disorders of the large and small vessels are common and serious complications of diabetes mellitus (DM). Studies over the last two decades have shown that hyperhomocysteinemia is associated with several macrovascular diseases, such as coronary artery disease, cerebrovascular disease, peripheral arterial disease, and deep-vein thrombosis [1–3].

Relatively few studies have investigated the association between hyperhomocysteinemia and diabetic retinopathy (DR) with yet inconsistent results. In addition, the vitamin status and its contribution to the development of DR have not been examined. In our study, we aimed to investigate whether there is an association between serum levels of homocysteine and those of folic acid and B-vitamins with DR in patients with type 2 diabetes mellitus (T2DM).

2. Materials and Methods

2.1. Subjects. The study group included 140 patients with T2DM, 69 men and 71 women, aged from 41 to 83 years (mean age 64.4 ± 9.9 years), all of Greek descent. All patients were recruited from the Ophthalmology Outpatient Clinics as well as the Diabetes Center of the "Attikon" University Hospital (Medical School of Athens University) in Athens, Greece. Diabetes was diagnosed according to the guidelines of the American Diabetes Association [4].

A detailed medical history was obtained from all study participants. The study included patients with known age of diagnosis of diabetes >40 years. We excluded patients with one or more of the following conditions:

(1) patients with a history of vascular disease (myocardial infarct or angina, stroke, peripheral arterial disease, and deep-venous thrombosis), renal, hepatic, chronic gastroenterologic, thyroid or blood disease, dementia, and neoplasm, since all these conditions are known to affect homocysteine blood concentrations;

(2) patients receiving vitamin supplementation or medications known to affect serum homocysteine concentrations, such as theophylline, statins, fibrates, levodopa, protons pump inhibitors, anticonvulsives, and contraceptives, and patients consuming good amounts of alcohol and heavy smokers;

(3) patients with uncontrolled arterial hypertension, defined as a systolic blood pressure ≥ 140 mmHg, and/or a diastolic blood pressure ≥ 90 mmHg;

(4) patients with uncertain diabetes duration.

The study was in accordance with the declaration of Helsinki and was approved by the Local Ethics Committee. Written informed consent was obtained from all subjects.

2.2. Methods

2.2.1. Ophthalmologic Examination. All patients underwent a complete ophthalmologic examination. Retinopathy was assessed by dilated ophthalmoscopy, fundus photography, and fluorescein angiography, when was indicated. Retinopathy was defined as the presence of any of such characteristic lesions: microaneurysms, hemorrhages, cotton wool spots, intraretinal microvascular abnormalities, hard exudates, venous beading, new vessels, laser scars, or a history of vitrectomy. Grading of the retinal findings was assessed according to the system used by the Early Treatment Diabetic Retinopathy Study Research Group [5]. Patients with diabetes were classified into two groups according to the findings of the "worst eye": 65 with DR (46.4%) and 75 without DR (53.6%). Of the 65 patients with DR, 36 (55.4%) had nonproliferative diabetic retinopathy (NPDR) and 29 (44.6%) had proliferative diabetic retinopathy (PDR).

2.2.2. Biochemical Analyses. Overnight fasting blood samples were taken from all subjects and were centrifuged within 1 hour following collection. The samples for homocysteine estimation were immediately chilled on ice and then were centrifuged and the serum was stored at −20°C. Serum glucose concentration was measured with a colorimetric assay on an automated analyzer MODULAR P800 (Roche Diagnostics GmbH, Mannheim, Germany) and glycosylated hemoglobin (HbA_{1C}) with an immunoturbidimetric assay on an analyzer INTEGRA 400plus (Roche Diagnostics GmbH, Mannheim, Germany). Serum creatinine concentration was measured with the colorimetric assay (Jaffé) on the automated analyzer MODULAR P800. Folic acid and vitamin B_{12} levels were estimated by electrochemiluminescence immunoassay on the analyzer MODULAR ANALYTICS E170 (Roche Diagnostics GmbH, Mannheim, Germany) and those for vitamins B_2 and B_6 by high performance liquid chromatography (HPLC) with fluorescence detection (Chromsystem Diagnostics GmbH, München, Germany). Estimation of serum homocysteine concentration was carried out by fluorescence polarization immunoassay (AXSYM SYSTEM, Abbott Diagnostics, Mannheim, Germany). Estimation of body weight is made by using the body mass index (BMI) = kg/height(m)^2.

2.3. Statistical Analysis. Differences between patients with and without DR in continuous variables were tested using *t*-test for two independent samples and, when normality assumption was violated, using the Mann-Whitney test. For categorical variables, chi square or Fisher's exact test was applied. Association of homocysteine with folic acid and vitamin B_{12} was investigated using Spearman correlation coefficient, while when HbA_{1C} and diabetes duration groups were considered, one-way ANOVA or Kruskal-Wallis test (with Bonferroni correction when needed) was used. Differences by gender and type of retinopathy were tested by *t*-test for two independent samples or the Mann-Whitney test. The association of homocysteine with possible prognostic variables was also tested in multivariable regression models. This was a two-stage procedure. In the first stage, using generalized additive models (GAMs), the shape of the association with continuous variables was explored. When a threshold was identified, this was estimated using appropriate methodology. In the final regression model, those variables identified in the first step were included in the model using two linear parts, one below and one above the threshold (piecewise linear). Finally, multivariable logistic regression models were used to identify prognostic variables for DR (binary variable, yes/no) in the same way that was described above for homocysteine.

3. Results

All our results are presented in median (interquartile range) values. The demographic and laboratory data of the study population are shown in Table 1. The age of patients with DR was statistically higher than that of patients without DR (68.0 (60.0–75.0) versus 61.0 (55.0–67.0), resp., $P = 0.001$), as was also their positive family history of diabetes (86.2% versus 54.7% resp., $P < 0.001$), and their BMI (29.2 (27.9–31.2 versus 27.8 (26.1–30.2), resp., $P = 0.004$). Patients with DR had a significantly longer diabetes duration (15.0 (10.0–22.0) versus 6.0 (3.0–9.0), $P < 0.001$), higher serum glucose (154.0 (126.0–188.0) versus 119.0 (103.0–136.0), $P < 0.001$), HbA_{1C} (7.4 (6.6–8.9) versus 6.7 (6.0–7.6), $P < 0.001$), and serum creatinine level (0.9 (0.8–1.1) versus 0.9 (0.7–1.0), $P = 0.024$), but in both groups creatinine levels were well within the normal range. A significantly higher serum homocysteine level was observed in patients with DR than in those without DR (16.3 (14.7–19.8) versus 11.1 (9.5–13.6), resp., $P < 0.001$), and hyperhomocysteinemia (serum homocysteine >15 μmol/L) was noted in 70.8% of individuals with DR compared to 14.7% without DR ($P < 0.001$). As for the serum vitamins, patients

Table 1: Demographic and laboratory characteristics of patients with type 2 diabetes mellitus.

Characteristics	DR− ($N = 75$)	DR+ ($N = 65$)	P value
Age (years)	61.0 (55.0–67.0)	68.0 (60.0–75.0)	0.001
Positive family history (%)	54.7 ($N = 41$)	86.2 ($N = 56$)	<0.001
BMI (kg/m^2)	27.8 (26.1–30.2)	29.2 (27.9–31.2)	0.004
Diabetes duration (years)	6.0 (3.0–9.0)	15.0 (10.0–22.0)	<0.001
Fasting serum glucose (mg/dL)	119.0 (103.0–136.0)	154.0 (126.0–188.0)	<0.001
HbA_{1C} (%)	6.7 (6.0–7.6)	7.4 (6.6–8.9)	<0.001
Serum creatinine (mg/dL)	0.9 (0.7–1.0)	0.9 (0.8–1.1)	0.024
Serum homocysteine (μmol/L)	11.1 (9.5–13.6)	16.3 (14.7–19.8)	<0.001
Hyperhomocysteinemia (%)	14.7 ($N = 11$)	70.8 ($N = 46$)	<0.001
Serum folic acid (ng/mL)	11.0 (8.3–17.0)	8.7 (7.2–10.3)	<0.001
Serum vitamin B_2 (μg/L)	10.8 (7.8–13.2)	9.1 (7.0–12.7)	0.179
Serum vitamin B_6 (μg/L)	11.6 (9.9–13.2)	11.0 (8.0–14.6)	0.481
Serum vitamin B_{12} (pg/mL)	361.5 (255.6–471.2)	288.8 (209.5–416.5)	0.014
Systolic blood pressure (mmHg)	125.0 (115.0–130.0)	130.0 (120.0–135.0)	0.016
Diastolic blood Pressure (mmHg)	75.0 (70.0–80.0)	75.0 (70.0–80.0)	0.409

Values are presented as median (interquartile range) or % (number).

Table 2: Association of serum homocysteine levels with diabetes duration and glycosylated hemoglobin.

Parameters		Homocysteine (μmol/L)	
Diabetes duration (years)	≤5 ($N = 42$)	11.2 (8.7–13.9)	P value*
	6–15 ($N = 60$)	12.2 (10.0–16.2)	
	≥16 ($N = 38$)	16.9 (14.8–18.9)	
	Overall ($N = 140$)	12.9 (10.3–16.9)	
HbA_{1C} (%)	<6.5 ($N = 43$)	11.2 (9.5–15.8)	P value**
	6.5–7.4 ($N = 43$)	12.3 (10.6–18.4)	
	7.5–8.4 ($N = 26$)	15.0 (11.1–16.2)	
	≥8.5 ($N = 28$)	15.1 (11.3–17.4)	
	Overall ($N = 140$)	12.9 (10.3–16.9)	

* ≤5 years versus ≥16 $P < 0.001$, 6–15 years versus ≥16 $P = 0.001$, overall $P < 0.001$.
** $P = 0.126$.
Values are presented as median (interquartile range).

with DR had, though in the normal range, significantly lower folic acid (8.7 (7.2–10.3) versus 11.0 (8.3–17.0), $P < 0.001$) and vitamin B_{12} (288.8 (209.5–416.5) versus 361.5 (255.6–471.2), $P = 0.014$) levels, whereas there was no significant difference for vitamin B_2 (9.1 (7.0–12.7) versus 10.8 (7.8–13.2), $P = 0.179$) and vitamin B_6 (11.0 (8.0–14.6) versus 11.6 (9.9–13.2), $P = 0.481$) levels between DR and no DR groups, respectively. Systolic blood pressure was higher in patients with DR (130.0 (120.0–135.0) versus 125.0 (115.0–130.0), $P = 0.016$), but no significant difference in diastolic blood pressure was noted between the two groups (75.0 (70.0–80.0) versus 75.0 (70.0–80.0), $P = 0.409$). However, in all our diabetic patients blood pressure was within the normal range.

In Table 2, the data on the association between serum homocysteine levels and two significant parameters are shown. Serum homocysteine levels of diabetic patients were significantly increased with increasing diabetes duration (≤5 versus ≥16 years, homocysteine: 11.2 (8.7–13.9) versus 16.9 (14.8–18.9), $P < 0.001$, 6–15 versus ≥16 years, homocysteine: 12.2 (10.0–16.2) versus 16.9 (14.8–18.9), $P = 0.001$, overall $P < 0.001$). However, serum homocysteine levels did not change significantly with increments in HbA_{1C} levels ($HbA_{1C} < 6.5$ homocysteine: 11.2 (9.5–15.8), 6.5–7.4: 12.3 (10.6–18.4), 7.5–8.4: 15.0 (11.1–16.2), ≥8.5: 15.1 (11.3–17.4), $P = 0.126$).

There was a statistically significant difference in serum homocysteine levels between the NPDR group compared to the PDR group (NPDR: 15.5 (11.8–17.4), PDR: 18.7 (16.5–22.0), $P = 0.001$).

A statistically significant negative linear relationship was found between serum homocysteine and folic acid levels (Spearman $r = -0.261$, $P = 0.002$), as well as between serum homocysteine and vitamin B_{12} levels (Spearman $r = -0.219$, $P = 0.009$).

Multiple linear regression analysis revealed a significant association between serum homocysteine level and age (Coef.: 0.099, 95% CI: 0.02, 0.18, $P = 0.018$), diabetes duration (Coef.: 0.106, 95% CI: 0.02, 0.20, $P = 0.020$), and an inverse relationship with folic acid (Coef.: −0.220, 95%

Table 3: Multiple linear regression analysis of the association of serum homocysteine with independent variables.

	Coef.	95% CI	*P* value
Age (years)	0.099	0.02, 0.18	0.018
Sex (male)	0.859	−0.56, 2.28	0.233
BMI (kg/m^2)	0.149	−0.06, 0.36	0.166
Fasting serum glucose (mg/dL)	0.005	−0.01, 0.02	0.523
Diabetes duration (years)	0.106	0.02, 0.20	0.020
Folic acid (ng/mL)	−0.220	−0.37, −0.07	0.005
Vitamin B_{12}[†] (pg/mL)	−0.027	−0.04, −0.01	0.003
Vitamin B_{12}[‡] (pg/mL)	−0.002	−0.01, 0.00	0.409

[†]below threshold.
[‡]above threshold.

Table 4: Multiple logistic regression analysis of the association of diabetic retinopathy with prognostic variables.

	OR	95% CI	*P* value
Age (years)	1.03	0.97–1.09	0.332
Diabetes duration (years)	1.18	1.08–1.28	<0.001
HbA_{1C} (%)	2.30	1.49–3.54	<0.001
Homocysteine[†] (μmol/L)	1.66	1.24–2.23	0.001
Homocysteine[‡] (μmol/L)	0.83	0.61–1.12	0.219
Folic acid (ng/mL)	0.73	0.62–0.86	<0.001

[†]above threshold.
[‡]below threshold.

CI: −0.37, −0.07, $P = 0.005$) and vitamin B_{12} (Coef.: −0.027, 95% CI: −0.04, −0.01, $P = 0.003$). No associations with sex (Coef.: 0.859, 95% CI: −0.56, 2.28, $P = 0.233$), BMI (Coef.: 0.149, 95% CI: −0.06, 0.36, $P = 0.166$), and glucose (Coef.: 0.005, 95% CI: −0.01, 0.02, $P = 0.523$) were observed (Table 3). There is a threshold in the exposure-response association of serum homocysteine with vitamin B_{12}, below which changes in serum vitamin B_{12} levels significantly affect serum homocysteine concentration (turning point: 248.4, standard error: 43.8) (Figure 1).

Multiple logistic regression analysis also showed that variables that independently affect DR risk were diabetes duration (OR: 1.18, 95% CI: 1.08, 1.28, $P < 0.001$), HbA_{1C} (OR: 2.30, 95% CI: 1.49, 3.54, $P < 0.001$), and homocysteine concentrations (OR: 1.66, 95% CI: 1.24, 2.23, $P = 0.001$). Folic acid conferred a protective effect on the DR risk (OR: 0.73, 95% CI: 0.62, 0.86, $P < 0.001$) (Table 4). There is a threshold in the association of homocysteine with DR (turning point: 13.7, standard error: 1.4). For every increase of serum homocysteine by 1 μmol/L above the threshold, there is an increased risk of about 66% for the development of DR (Figure 2).

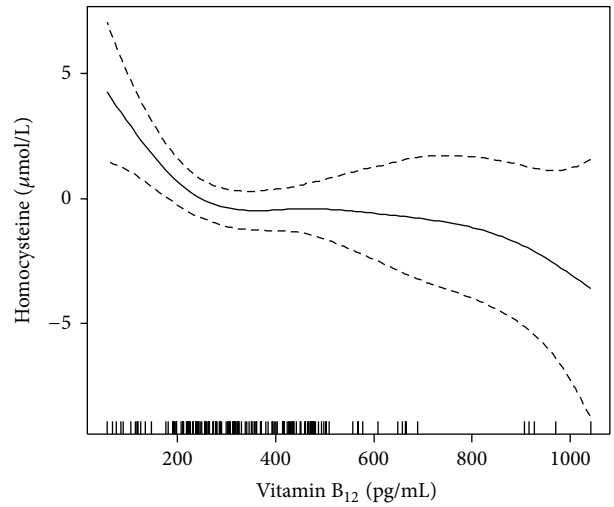

Figure 1: Shape of association between serum homocysteine and B_{12}.

4. Discussion

The results of our study revealed significantly higher concentration of serum homocysteine as well as a higher prevalence of hyperhomocysteinemia (serum homocysteine > 15 μmol/L) in patients with T2DR compared to those without DR and hyperhomocysteinemia as an independent risk factor for DR. Our results also revealed a threshold in the association of serum homocysteine level with DR risk of around 14.0 μmol/L, above which a 1 μmol/L increase in serum homocysteine concentration was an independent predictor of increased DR risk of 66%. A higher serum homocysteine level in patients with PDR than in those with NPDR was also found; a result that may suggest that homocysteine is related not only to the development but also to the progression of DR. There are a number of studies that have evaluated the association between hyperhomocysteinemia and type 2 DR but have so far yielded inconsistent

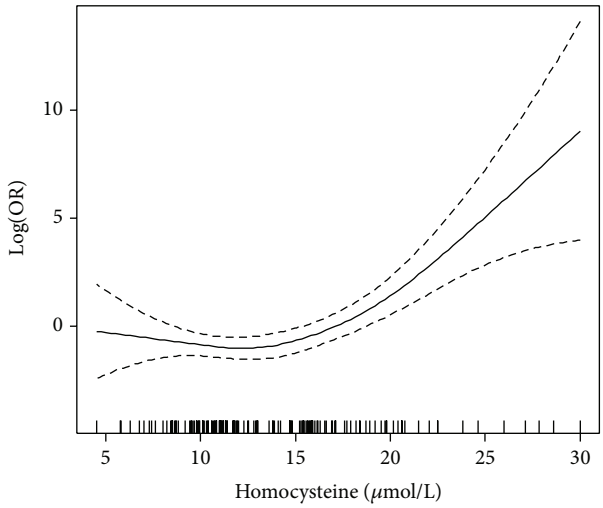

Figure 2: Shape of association between DR risk and serum homocysteine.

results [6–9]. The differences in the findings among studies are most likely related to differences in type of diabetes or to inadequately controlling for confounding factors, such as nephropathy and hypertension, but also lifestyle (e.g., vitamin intake) and genetic factors. In addition, the cutoff level for hyperhomocysteinemia differed substantially among studies (from 11.0 to 16.0 μmol/L).

The exact pathogenesis of DR is multifactorial and remains largely unclear but may involve (1) endothelial dysfunction and (2) low-grade chronic inflammation of the retinal capillaries. Hyperhomocysteinemia promotes these two pathophysiologic mechanisms [10]. It has long been recognized that oxidative stress is associated with the progression of diabetes and its complications. The adverse effects of hyperhomocysteinemia on the endothelium may be triggered by increased oxidative stress in the diabetic vasculature. Hyperhomocysteinemia increases NADPH oxidase activity [11], promotes uncoupling of endothelial nitric oxide synthase [12], and inhibits the function of intracellular antioxidant enzymes, such as glutathione peroxidase and superoxide dismutase [13]. Moreover, autooxidation of excess homocysteine may directly lead to additional ROS production [14]. Accumulating ROS reacts with nitric oxide (NO) to form peroxynitrite radicals, leading to decreased NO bioavailability and activity and subsequent endothelial dysfunction.

ROS activate inflammatory mediators and cytokines (interleukins, tumor necrosis factor-α). In addition, upregulation of ROS leads to activation of proinflammatory mediators, such as the nuclear factor-κB, in retinal capillary cells, which further increasing the expression of cytokines [15]. These inflammatory events result in increased endothelial cell expression of adhesion molecules, which contribute to leucocyte accumulation and attachment to the retinal capillaries (leucostasis) [16]. Leucostasis, which is believed to be an early event in DR, may lead to the blood-retinal barrier breakdown and eventually to chronic leucocyte mediated cell damage and death [17].

With regard to the risk factors for DR, regression analysis of our data confirmed that poor glycemic control and longer diabetes duration are independent risk factors for the development and progression of DR [18, 19]. In our study, the group of patients without DR succeeded in achieving the target mean HbA_{1C} level of 7.0% or less [4] (HbA_{1C}6.9±1.1%) but this was not the case with the group of patients with DR (HbA_{1C} 7.8 ± 1.5%). However, it is true that perfect glycemic control is very difficult to attain for most diabetic patients and glucose control has the tendency to deteriorate with time. A significant finding in our study was the association of increasing serum homocysteine concentration with longer diabetes duration. Thus, we may infer that the known harmful effect of DM duration on the development of retinopathy and its progression is not only related to the long-standing hyperglycemia but also to the increased homocysteine concentrations with time and its subsequent adverse effects on retinal capillaries. On the contrary, we did not find an association between homocysteine concentrations and increasing HbA_{1C} levels. This may be due either to an actual lack of such an association between the two factors or to the inconsistency of hyperglycemia during the progression of DM. Besides, evidence from both healthy and diabetic twins indicates that HbA_{1C} levels are determined for a significant part by genetic factors. This provides evidence that HbA_{1C} is in part determined by factors other than glycemic control and may account for the variation in HbA_{1C} levels among people [20, 21].

Methionine and homocysteine metabolism depends upon adequate stores of folic acid and vitamins B_2, B_6, B_{12}, which act as cofactors or substrates in the metabolism and are important nutritional determinants of serum homocysteine. A number of studies have shown an inverse association between blood folic acid and homocysteine concentration [22, 23]. Such an inverse relationship between folic acid and homocysteine levels was also observed in our study. In regard with vitamin B_{12}, the reason for the limited number of studies with this vitamin is that its deficiency is rather unusual, especially in Western countries, due to the fact that the vitamin is present in food of animal origin. A recent study from India, where the prevalence of micronutrient deficiency is reported to be high, mentions a high prevalence of vitamin B_{12} deficiency in the diabetic population (54–67%). Mean vitamin B_{12} level in the DR group was below the normal range and higher homocysteine levels were significantly associated with lower vitamin B_{12} levels [24]. We also detected such an inverse association between vitamin B_{12} and homocysteine levels. Our study illustrates lower levels of folic acid and vitamin B_{12} in patients with DR. It is noteworthy that low, but not necessarily deficient, levels of these two vitamins contributed to the development of hyperhomocysteinemia in our study. Regression analysis also showed that folic acid confers a protective effect on the development of DR. Another significant observation in our study was the finding of a threshold of about 250 pg/mL of serum vitamin B_{12}, below which there is a sharp rise in serum homocysteine with decreasing vitamin B_{12} levels.

Vitamin B_6 deficiency is extremely unusual, because this vitamin is present in a wide range of food of animal and plant

origin. In our study, we found no cases of biochemical vitamin B_6 deficiency. One study has reported an association between low vitamin B_6 and vascular disease that was unrelated to hyperhomocysteinemia, but it was explained by a relationship between low vitamin B_6 status and chronic inflammation, which is known to promote DR [25]. The results of our study show that vitamin B_6 levels were well within the normal range in patients with diabetes irrespective of the presence of retinopathy. Vitamin B_2 acts in the form of flavin adenine dinucleotide (FAD), which is a substrate for the enzyme methylene tetrahydrofolate reductase (MTHFR). This enzyme is the most important genetic determinant of blood homocysteine in the general population. In our study material, no cases of biochemical vitamin B_2 deficiency were observed, neither do we know any such studies, and the levels of this vitamin were also well within the normal range in all of our diabetic patients. Increased dietary folic acid and vitamin B_2 requirements have been observed mainly in persons with the genetic variant of the enzyme MTHFR, C677T [26]. Studies confirm that vitamin B_2 is an independent determinant of homocysteine in TT homozygotes of the C677T polymorphism [27].

In conclusion, among the many factors, some of them are yet unidentified, that affect the development and progression of DR, hyperhomocysteinemia seems to be associated with DR, at least as a biomarker. Longer diabetes duration and lower folic acid and vitamin B_{12} status appear to be important determinants for hyperhomocysteinemia in DR patients. Monitoring serum homocysteine concentration, as well as folate and vitamin B_{12} status in T2DM patients, could be used as an indicator for assessing microvascular risk in DM. Treatment of existing hyperhomocysteinemia with folic acid and vitamin B_{12} may be useful in reducing the risk of microvascular complications in T2DM.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Effects of Simvastatin on Glucose Metabolism in Mouse MIN6 Cells

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The aim of this study was to investigate the effects of simvastatin on insulin secretion in mouse MIN6 cells and the possible mechanism. MIN6 cells were, respectively, treated with 0 μM, 2 μM, 5 μM, and 10 μM simvastatin for 48 h. Radio immunoassay was performed to measure the effect of simvastatin on insulin secretion in MIN6 cells. Luciferase method was used to examine the content of ATP in MIN6 cells. Real-time PCR and western blotting were performed to measure the mRNA and protein levels of inward rectifier potassium channel 6.2 (Kir6.2), voltage-dependent calcium channel 1.2 ($Ca_v1.2$), and glucose transporter-2 (GLUT2), respectively. ATP-sensitive potassium current and L-type calcium current were recorded by whole-cell patch-clamp technique. The results showed that high concentrations of simvastatin (5 μM and 10 μM) significantly reduced the synthesis and secretion of insulin compared to control groups in MIN6 cells ($P < 0.05$). ATP content in simvastatin-treated cells was lower than in control cells ($P < 0.05$). Compared with control group, the mRNA and protein expression of Kir6.2 increased with treatment of simvastatin ($P < 0.05$), and mRNA and protein expression of $Ca_v1.2$ and GLUT2 decreased in response to simvastatin ($P < 0.05$). Moreover, simvastatin increased the ATP-sensitive potassium current and reduced the L-type calcium current. These results suggest that simvastatin inhibits the synthesis and secretion of insulin through a reduction in saccharometabolism in MIN6 cells.

1. Introduction

Statins are inhibitors of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase that can reduce the plasma level of total cholesterol, low density lipoprotein cholesterol, and glycerin trilaurate and increase high density lipoprotein cholesterol [1]. In the past two decades, more than 170,000 participants in a number of large-scale clinical trials demonstrated that statins reduce the incidence of cardiovascular events [2]; thus, statins are now widely used for the primary and secondary prevention of both atherosclerotic cardiovascular disease (ASCVD) and stroke [3–6]. However, in contrast to its clear benefit to cardiovascular disease prevention, a substantial number of meta-analyses of large, randomized controlled clinical trials showed that statins also increased the risk of new onset diabetes, in comparison to the placebo group [7–9]. The mechanism by which statins cause this increase in type 2 diabetes risk is unknown. Accordingly, in this study, we investigated the effects of statins on the two known mechanisms that cause type 2 diabetes: insulin resistance and the dysfunction of islet beta cells. Thus, we tested the effects of simvastatin on insulin secretion in the mouse islet beta cell line, MIN6, and explored the mechanism of this function.

2. Materials and Methods

2.1. Reagents. The mouse islet beta cell line, MIN6, was a gift from the Ruijin Hospital, affiliated to Shanghai Jiao Tong University School of Medicine, Institute of Endocrinology. Simvastatin was obtained from MERCK; antibodies against inward rectifier potassium channel 6.2 (Kir6.2) and GLUT2, as well as the HRP-conjugated rabbit polyclonal

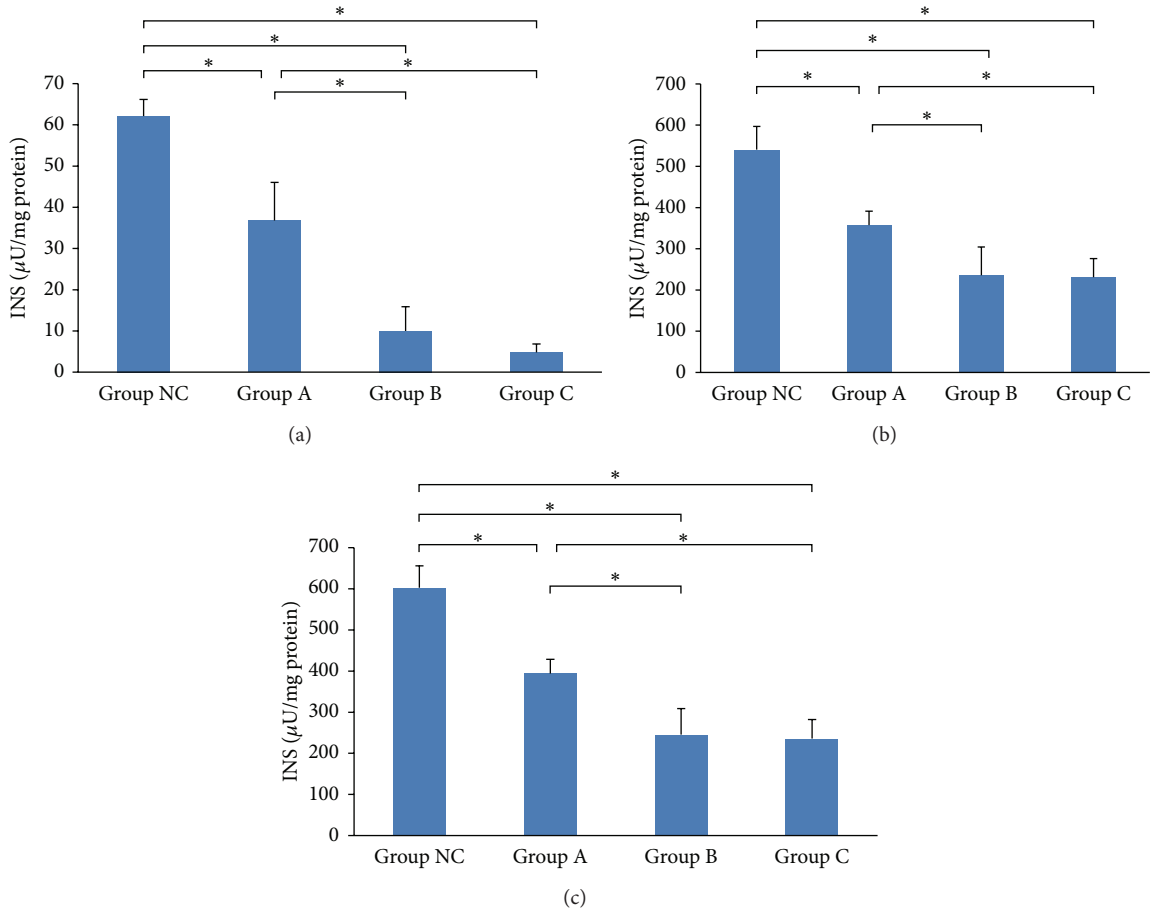

Figure 1: Effects of simvastatin on insulin content at low glucose levels. (a) Insulin secretion. (b) Insulin content in MIN6 cells. (c) Total amount of insulin. INS indicates insulin-releasing test. $^{*}P < 0.05$.

antibody, were purchased from Abcam. The antibody against $Ca_v1.2$ was obtained from Novus; the antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Cell Signaling. The ATP detection assay kit was obtained from the Beyotime Institute of Biotechnology.

The formulation of the extracellular fluid for the K_{ATP} channel currents (I_{KATP}) was as follows: 80 mM NaCl; 60 mM KCl; 10 mM HEPES; 1 mM $MgCl_2$; 0.1 mM $CaCl_2$ (pH 7.4, NaOH). The electrode internal fluid for these K_{ATP} channel currents contained the following: 15 mM NaCl; 92 mM KCl; 33 mM KOH; 10 mM HEPES; 1 mM $MgCl_2$; 1 mM $CaCl_2$; 10 mM EGTA; 0.2 mM MgATP (pH 7.2, KOH) [1].

The formulation of the extracellular fluid for the L-Ca channel currents ($I_{Ca\text{-}L}$) was as follows: 30 mM $BaCl_2$; 99.3 mM CsCl; 16.7 mM glucose; 10 mM tetraethylammonium chloride (TEACl); 1.2 mM $MgCl_2$; 10 mM Hepes (pH 7.4, CsOH). The electrode internal fluid for these L-Ca channel currents contained the following: 130 mM CsCl; 10 mM TEACl; 5 mM $MgCl_2$; 4 mM ATP-Na_2; 0.4 mM GTP-Na_2; 10 mM EGTA; 10 mM Hepes (pH 7.2, CsOH) [2].

All solutions were filtered through a microporous membrane (0.22 μm).

2.2. Cell Culture. MIN6 cells were cultured in DMEM supplemented with 15% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 70 μmol/L beta-mercaptoethanol. Cells were divided into NC, A, B, and C groups at 6×10^5/mL; medium was changed every two days, and A, B, and C groups were treated with 2, 5, and 10 μM simvastatin, respectively, for 48 h. NC was the normal control group.

2.3. Insulin-Releasing Test and Detection of Insulin Content in MIN6 Cells. Cells were cultured in 12 wells at 5×10^4/mL for 48 h and then washed with KRBB (115 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 24 mM $NaHCO_3$, 0.1% BSA, and 10 mM Hepes, pH 7.4) twice; glucose-free Krebs-Ringer buffer was added for 2 h at 37°C, and then the medium was removed. Krebs-Ringer buffer with 2.8 mM and 16.7 mM was added into each group for 1 h at 37°C; then the supernatant was removed, and the cells were centrifuged at 1000 rpm for 5 min at 4°C; then the supernatant was used for immediate testing or stored at −70°C. Equal acid alcohol (75% ethanol, 1.5% hydrochloric acid, and 75% ultrapure water) was added to extract the cells, and then they

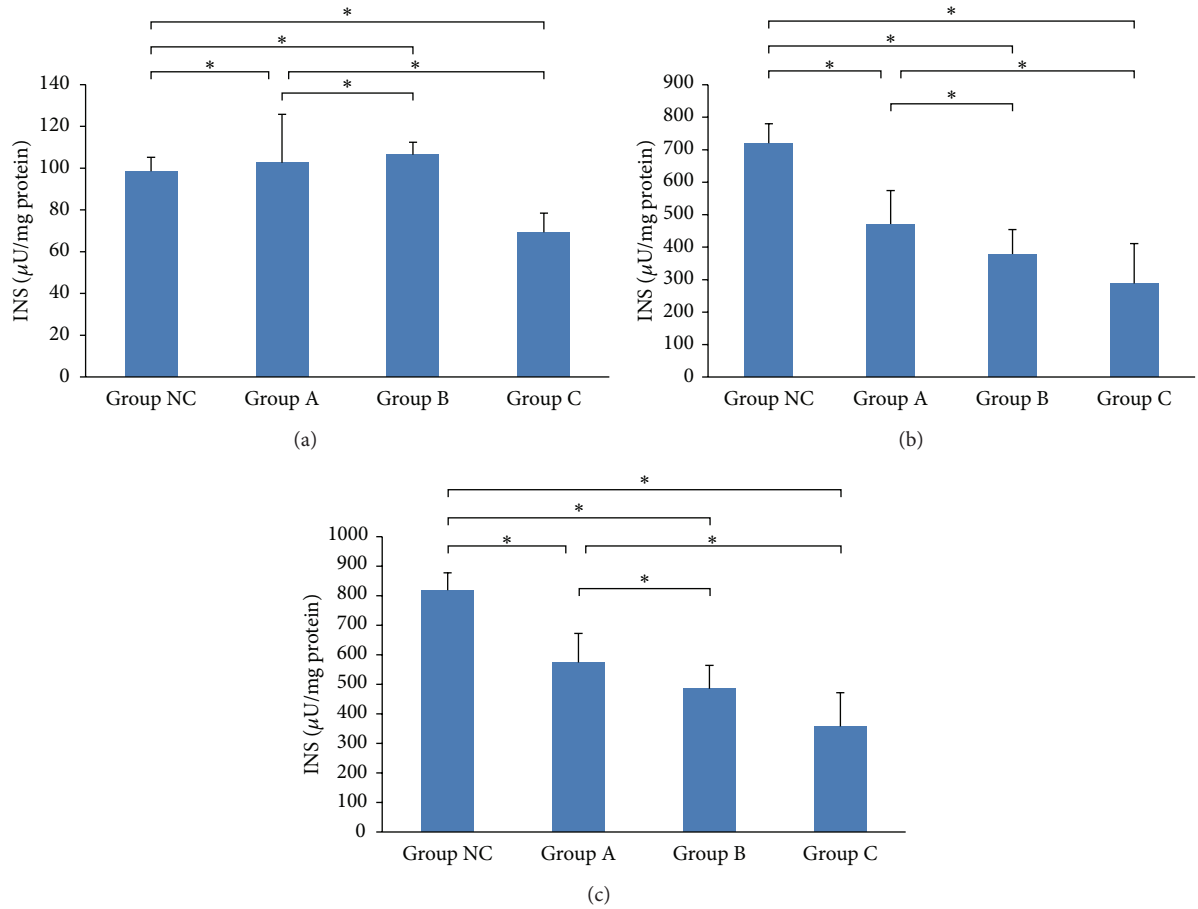

Figure 2: Effects of simvastatin on insulin content at high glucose level. (a) Insulin secretion. (b) Insulin content in MIN6 cells. (c) Total amount of insulin. INS indicates insulin-releasing test. $^{*}P < 0.05$ and $^{**}P < 0.01$.

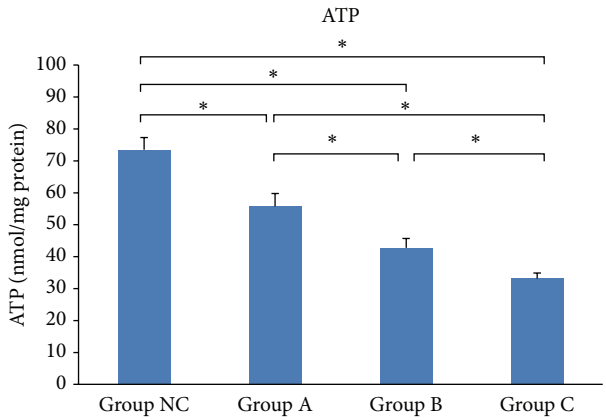

Figure 3: Effects of simvastatin on ATP levels in MIN6 cells. $^{*}P < 0.05$.

were stored at 4°C. In the following day, insulin was measured in the supernatant, according to the kit, and the results were adjusted with total protein concentration. Each experiment was repeated three times.

2.4. Measurement of ATP Content in MIN6 Cells. Cells were cultured in 12 wells; 100 μL cell lysis buffer was added to each well, and then the lysed cells were centrifuged at 12,000 g for 8 min. The supernatant was collected; 100 μL ATP working solution was added to the tube at room temperature for 5 min, and then 20 μL sample buffer was added and mixed quickly; then the RLU was read with a luminometer.

2.5. Real-Time PCR. Total RNA was isolated using the Trizol reagent, according to the manufacturer's instructions. The cDNA was synthesized from 2 μg of total RNA, using MMLV transcriptase with random primers. Real-time PCR was performed using SYBR Premix ExTaq. Quantification was normalized to the amount of endogenous GAPDH. Primers used for real-time PCR are listed in Table 1.

2.6. Western-Blot Analysis. Cells were lysed with lysis buffer (200 mM Tris-HCl (pH 7.5), 1.5 M NaCl, 10 mM EDTA, 25 mM sodium pyrophosphate, 10 mM glycerol phosphate, 10 mM sodium orthovanadate, 50 mM NaF, and 1 mM PMSF, in combination with a protein inhibitor cocktail). 30 μg of protein lysates from each sample was subjected to SDS-PAGE

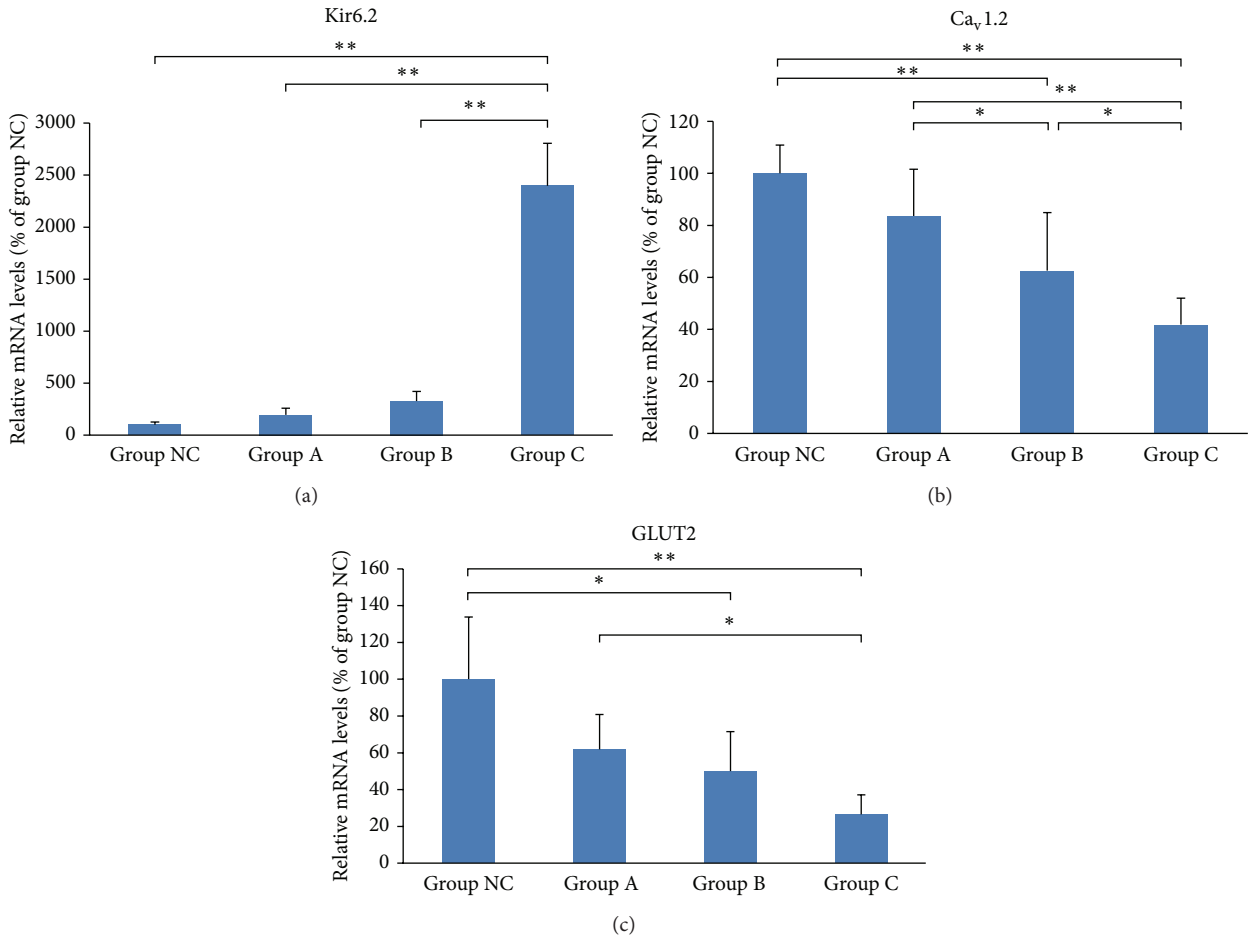

FIGURE 4: Effects of simvastatin on mRNA levels of Kir6.2 (a), $Ca_v1.2$ (b), and GLUT2 (c) in MIN6 cells. $^{*}P < 0.05$ and $^{**}P < 0.01$.

and transferred onto nitrocellulose membranes. Blots were incubated with the specific primary antibodies overnight at 4°C. After washing them three times for 15 min each with TBST (TBS + 0.1% Tween20), blots were incubated with a horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA) and visualized by chemiluminescence. Band densities were quantified by densitometry using the Scion Image software and normalized to β-actin levels.

2.7. I_{KATP} and I_{Ca-L} Recordings Using the Whole-Cell Patch-Clamp Technique. A coverslip containing adherent cells was mounted in a recording chamber on an Olympus IX 51 microscope (Olympus, Tokyo, Japan). Glass capillaries (Sutter, USA) were used to fabricate patch electrodes through a micropipette puller (P97, Sutter, USA), which had resistances of 2 to 5 MΩ, when filled with an intracellular solution. The cells were washed with extracellular solution for 2 min, and the patch electrodes, which were attached to the MIN6 cell (well-grown, smooth-faced, and tile-shaped), were moved using a motorized micromanipulator (MP-225, Sutter, USA). First, the liquid junction potential was eliminated; then the electrode capacitance transients were compensated, and, after gigaseals (>1 GΩ), fast capacitance was established by negative pressure. Using further suction to break the membrane, a whole-cell recording was formed. Finally, slow capacitance was compensated. Membrane capacitance around 7~9 pF was selected as the last record.

The I_{KATP} was elicited from a holding potential of −70 mV, with a prepulse to −40 mV for 500 ms, and with test potentials ranging from −120 mV to +80 mV in 20 mV increments during 3000 ms. The I_{Ca-L} was elicited from a holding potential of −70 mV, a prepulse to −70 mv for 500 ms, and depolarized from −40 to +50 mV in 10 mV increments during 3000 ms. Calcium currents were recorded within 5–20 minutes.

All recordings were performed at room temperature (~23°C), with a 100 μs sampling interval, and digitally filtered at 1 kHz. Leak subtraction for gating currents was performed using the P/n protocol, as implemented in patch machine. All patch-clamp data were acquired with a patch-clamp amplifier (AXON 700B, USA), digitized with a Digidata 1440A analog-digital converter (Molecular Devices, USA), and analyzed with pClamp9+clampfit software.

2.8. Statistical Analysis. Data were collected from several independent experiments, with three replicates per experiment. Quantitative data are expressed as mean ± standard deviation (SD). Data were analyzed using one-way ANOVA with Tukey's posttest or nonparametric tests in SPSS 15.0.

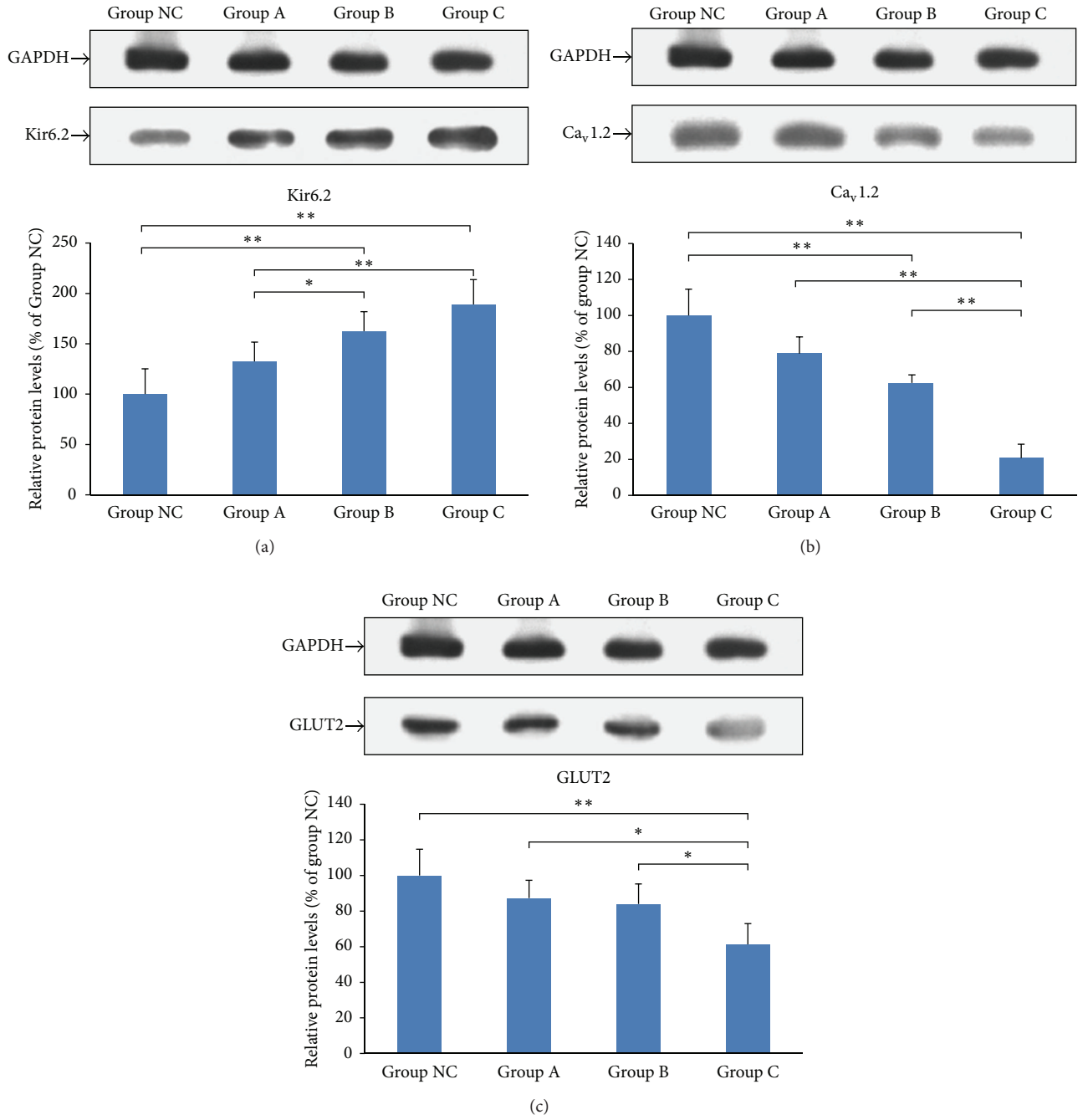

Figure 5: Effects of simvastatin on protein levels of Kir6.2 (a), $Ca_v1.2$ (b), and GLUT2 (c) in MIN6 cells. $^{*}P < 0.05$ and $^{**}P < 0.01$.

$P < 0.05$ was considered statistically significant. Bars in the graph represent SD.

3. Results

3.1. The Effects of Simvastatin on Insulin Content and Secretion in MIN6 Cells. Comparing with the normal control cells, the cells treated with simvastatin had less insulin content, less insulin secretion, and less total amount of insulin induced from a low level of glucose ($P < 0.05$). Furthermore, simvastatin decreased insulin secretion in a concentration-dependent manner (Table 2 and Figure 1).

Comparing with the normal MIN6 cells, cells treated with simvastatin had less insulin content, less insulin secretion, and less total amount of insulin induced at high glucose levels ($P < 0.05$). Furthermore, simvastatin reduced the total amount of insulin in a concentration-dependent manner. The group treated with 10 μM simvastatin had a greater decrease in cellular insulin content, insulin secretion, and total insulin, in comparison to the group treated with 2 μM simvastatin under high glucose levels ($P < 0.05$, Table 3 and Figure 2).

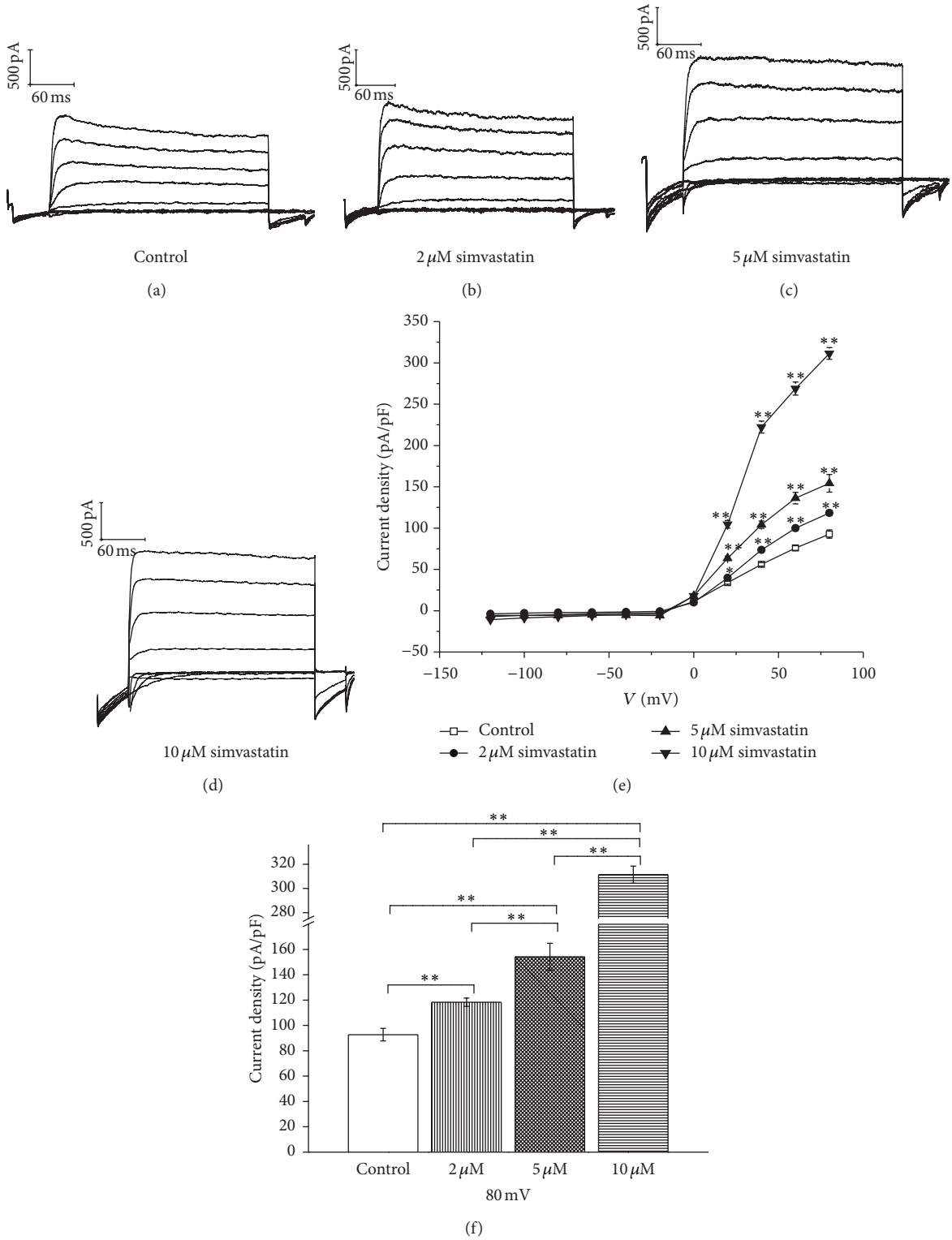

FIGURE 6: Effects of simvastatin on I_{KATP} and current density in MIN6 cells ($^{*}P < 0.05$, $^{**}P = 0.03$, and $^{***}P < 0.01$). (a) KATP current of control group ($n = 12$), (b) KATP current with application of 2 μmol/L simvastatin ($n = 12$), (c) KATP current with application of 5 μmol/L simvastatin ($n = 10$), (d) KATP current with application of 10 μmol/L simvastatin ($n = 8$), (e) current density-voltage (J-V) curves of four groups, and (f) the current densities of cells with application of different concentrations of simvastatin at 80 mV voltage.

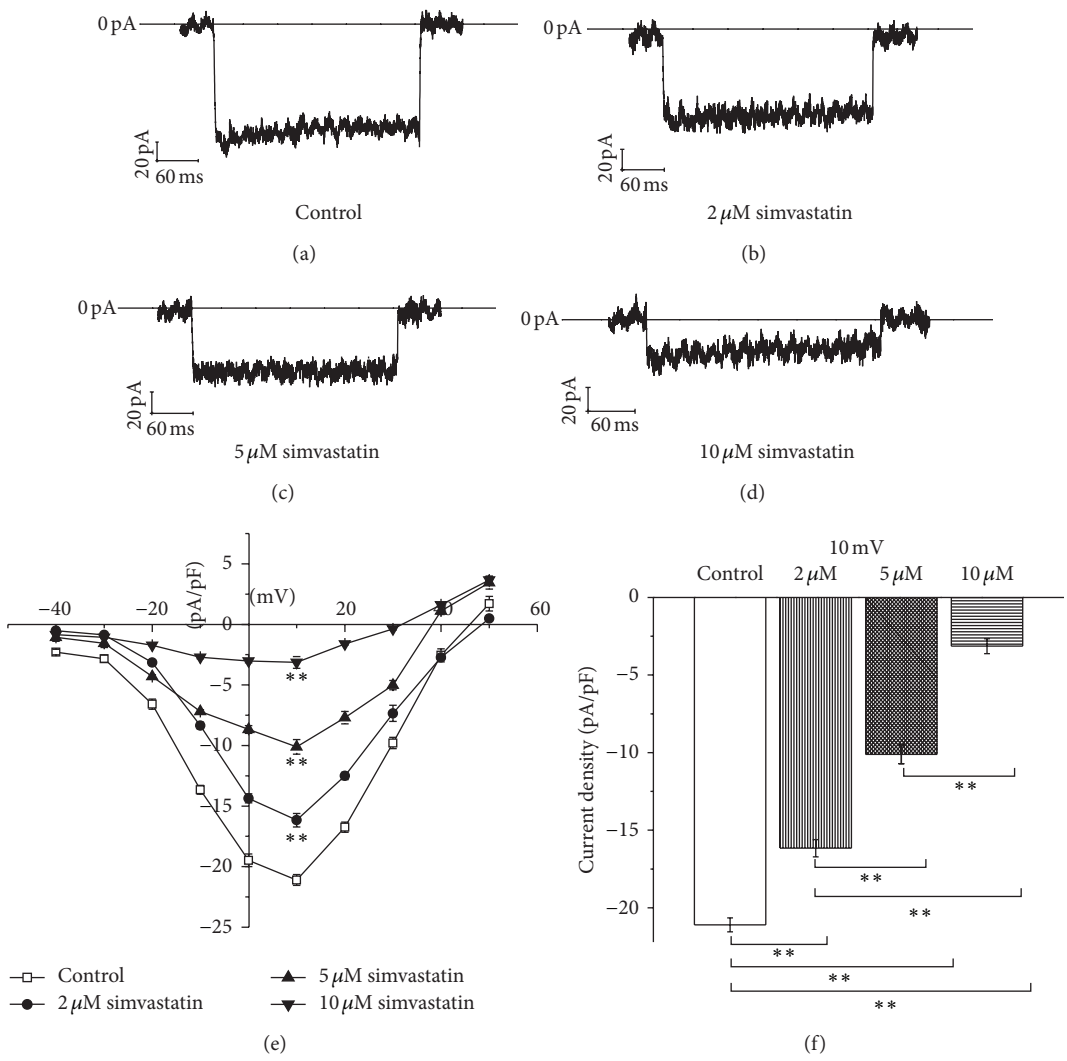

Figure 7: Effects of simvastatin on $I_{Ca\text{-}L}$ and current density in MIN6 cells. (a) $I_{Ca\text{-}L}$ current of control group cells at 10 mV, (b) $I_{Ca\text{-}L}$ current of cells with application of 2 μmol/L simvastatin at 10 mV, (c) $I_{Ca\text{-}L}$ current of cells with application of 5 μmol/L simvastatin at 10 mV, (d) $I_{Ca\text{-}L}$ current of cells with application of 10 μmol/L simvastatin at 10 mV, (e) current density-voltage (J-V) curves of four groups, and (f) the $I_{Ca\text{-}L}$ current densities of cells with application of different concentrations of simvastatin at 10 mV voltage.

Table 1: Real-time PCR primers.

Gene	Forward primers	Reverse primers
Kir6.2	AAGGGCATTATCCCTGAGGAA	TTGCCTTTCTTGGACACGAAG
$Ca_v1.2$	AGACGCTATGGGCTATGA	AACACCGAGAACCAGATTTA
GLUT2	TCAGAAGACAAGATCACCGGA	GCTGGTGTGACTGTAAGTGGG
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

Table 2: Effects of simvastatin on insulin content, secretion in MIN6 cells, and total insulin at low glucose levels (mean ± SD, $n = 6$, μIU/mg protein).

Group	Insulin secretion	Insulin content	Total amount of insulin
Group NC	62.12 ± 4.03	540.32 ± 56.75	602.43 ± 53.41
Group A	36.82 ± 9.20	357.17 ± 34.09	393.98 ± 34.33
Group B	10.00 ± 5.90	235.53 ± 68.70	245.53 ± 63.44
Group C	4.78 ± 2.05	231.02 ± 45.13	235.80 ± 46.36
F value	80.16	30.26	46.04
P value	<0.001	<0.001	<0.001

Table 3: Effects of simvastatin on insulin content, secretion in MIN6 cells, and total insulin at high glucose levels (mean ± SD, $n = 6$, μIU/mg protein).

Groups	Insulin secretion	Insulin content	Total amount of insulin
Group NC	98.53 ± 6.69	720.17 ± 59.66	818.69 ± 58.78
Group A	102.63 ± 23.13	471.09 ± 103.48	573.72 ± 98.43
Group B	106.49 ± 5.91	378.40 ± 75.83	484.88 ± 79.05
Group C	69.24 ± 9.25	288.29 ± 122.58	357.53 ± 114.43
F value	6.58	15.81	18.67
P value	0.007	<0.001	<0.001

Table 4: Effects of simvastatin on ATP levels in MIN6 cells.

Groups	ATP level (nmol/mg protein)	N
Group NC	73.49 ± 3.84	10
Group A	55.72 ± 4.06	10
Group B	42.79 ± 2.89	10
Group C	33.11 ± 1.75	10
F value	285.91	
P value	<0.001	

3.2. The Effects of Simvastatin on ATP Levels in MIN6 Cells. ATP levels decreased in MIN6 cell lysates after simvastatin treatment, in comparison to the normal control cell lysates ($P < 0.05$). Furthermore, simvastatin caused this decrease in ATP in a concentration-dependent manner ($P < 0.05$, Table 4 and Figure 3).

3.3. The mRNA Levels of Kir6.2, $Ca_v1.2$, and GLUT2. Compared with the normal control cells, the mRNA levels of Kir6.2 increased (23.97 ± 4.09, $P < 0.001$) in cells treated with 10 μM simvastatin, while the mRNA levels of $Ca_v1.2$ and GLUT2 decreased (0.63 ± 0.22, $P = 0.002$ and 0.50 ± 0.21, $P = 0.023$, resp.) in cells treated with 5 μM simvastatin and were further reduced in cells treated with 10 μM simvastatin (0.42 ± 0.10, $P < 0.001$ and 0.26 ± 0.11, $P < 0.001$, resp.). Kir6.2 mRNA levels were significantly higher in the 10 μM treatment group than in the 2 μM and 5 μM groups ($P < 0.01$). The $Ca_v1.2$ mRNA levels were significantly lower in the 10 μM treatment group than in the 2 μM and 5 μM groups ($P < 0.001$, $P = 0.038$). Furthermore, Ca_v 1.2 mRNA levels were more significantly downregulated in the 5 μM group than in the 2 μM group ($P = 0.036$). GLUT2 mRNA levels were more significantly reduced in the 10 μM group than in the 2 μM group ($P = 0.013$, Figure 4).

3.4. Protein Expression of Kir6.2, $Ca_v1.2$, and GLUT2. As shown in Figure 5, 5 μM and 10 μM simvastatin increased the protein expression of Kir6.2, while the expression of $Ca_v1.2$ decreased, in comparison to the control MIN6 cells. The protein expression of GLUT2 decreased in cells treated with 10 μM simvastatin, in comparison to the control MIN6 cells.

3.5. The Effect of Simvastatin on I_{KATP} in MIN6 Cells. Simvastatin increased I_{KATP} in a concentration-dependent manner. At 80 mV electric voltage, I_{KATP} increased by 118.36 ± 3.30 pA/pF, 154.16 ± 10.72 pA/pF, and 311.37 ± 7.06 pA/pF at 2 μM, 5 μM, and 10 μM simvastatin, respectively (Figure 6).

3.6. The Effect of Simvastatin on I_{Ca-L} in MIN6 Cells. As shown in Figure 7, at 10 mV, simvastatin significantly decreased the $I_{Ca\text{-}L}$ current density in a concentration-dependent manner. The current densities were −16.16 ± 0.56 pA/pF, −10.11 ± 0.59 pA/pF, and −4.71 ± 0.19 pA/pF in MIN6 cells treated with 2 μM, 5 μM, and 10 μM simvastatin.

4. Discussion

Statins inhibit cholesterol synthesis, reduce plasma cholesterol levels to stabilize the atherosclerotic plaque in blood vessels, and inhibit the activity of HMG-CoA reductase in the cholesterol synthesis process. Statins are widely used in a great number of medical fields, including cardiology, neurology, nephrology, and endocrinology. Recently, there has been a great deal of attention on the effect of statins, especially in enhancer dosages, on glycometabolism, but there have been few studies on the mechanism of how statins increase diabetes risk.

The mechanism of statins on glycometabolism, decreased insulin sensitivity, and diabetes risk increase is still unknown. Nakata et al. [10] found that atorvastatin reduces insulin sensitivity by inhibiting adipose cell maturation and the expression of glucose transporter-4. The side effect of statins on glycometabolism may be related to a decrease in metabolite production. Coenzyme Q10 plays a critical role in energy metabolism; it not only is important for antioxidation and cell membrane stabilization, but also participates in mitochondrial oxidative phosphorylation [11]. Some statins have been shown to significantly reduce plasma coenzyme Q10 level, leading to a reduction in mitochondrial oxidative phosphorylation and decreased ATP production [12–15]. Furthermore, statins can inhibit the mevalonate pathway, thus preventing the production of mevalonate metabolites, such as isoprenoid and coenzyme Q10, and affecting glycometabolism and insulin sensitivity.

Insulin synthesis and secretion are a complete and orderly process in pancreatic islet beta cells. At present, the consensus on glucose-stimulated insulin secretion is that beta cells are electrically excitable; GLUT2 transports glucose into the cells, and then glucokinase phosphorylates it to ATP. These events result in ATP-sensitive potassium channel (K_{ATP}) closure

and membrane depolarization, voltage-dependent calcium channel (Ca_v) opening, calcium influx, intracellular calcium concentration increase, and activation of a series of enzymes and proteins, which are necessary for insulin secretion. Therefore, many factors and stages affect insulin secretion in beta cells. Our study shows that, after 48 h of treatment, simvastatin significantly inhibited glucose-stimulated insulin synthesis and secretion at basal (2.8 mM) and high glucose levels (16.7 mM), in a dose-dependent manner in MIN6 cells.

In this study, we investigated the mechanism of how simvastatin inhibits insulin secretion in islet beta cells. ATP is a very important regulator of insulin secretion in islet beta cells; reduction of ATP inhibits the K_{ATP} channel closure, membrane depolarization, and subsequent Ca_v channel opening, thus inhibiting insulin secretion. Our results demonstrated that simvastatin significantly inhibited ATP production in a dose-dependent manner in islet beta cells.

K_{ATP} is an octamer, which consists of four Kir6.2 subunits and four thiourea receptor-1 (SUR1) subunits. K_{ATP} plays a critical role in the insulin secretion process by coupling glucose metabolism and electrical activity in beta cells [16]. The effects of K_{ATP} are dependent on the expression of Kir6.2 and SUR1 [17]. In addition, Ca_v, in its relation to the L-type non-L-type calcium channels, plays an important role in insulin secretion.

Our study showed that simvastatin significantly increased Kir6.2 expression, while it decreased $Ca_v1.2$ and GLUT2 expression at 5 μM and 10 μM treatment for 48 h. The patch-clamp technique results showed that simvastatin increased the K_{ATP} current, while it reduced the L-Ca current in MIN6 cells. Kir6.2 is an inward rectifier potassium channel, which plays important roles in membrane resting potential maintenance and insulin secretion in beta cells. Thus, by increasing Kir6.2, while decreasing $Ca_v1.2$, and subsequently inhibiting depolarization and calcium influx, simvastatin is able to reduce insulin secretion. Furthermore, simvastatin can also indirectly inhibit insulin secretion by decreasing GLUT2 expression, resulting in reduced glucose uptake.

Collectively, although it is clear that statins have beneficial effects on cardiovascular disease prevention, our study supports the evidence that long-term use of large doses of statins has adverse effects on glucose metabolism and insulin sensitivity. Therefore, patient blood glucose levels should be monitored to reduce these adverse events caused by statins.

5. Conclusions

This study has notable strengths. We found that simvastatin inhibits the synthesis and secretion of insulin through a reduction in saccharometabolism in beta cells.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jieqiong Zhou, Weihua Li, and Qiang Xie contributed equally to this work as first author.

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Research Article

Insulin Increases Ceramide Synthesis in Skeletal Muscle

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Aims. The purpose of this study was to determine the effect of insulin on ceramide metabolism in skeletal muscle. *Methods*. Skeletal muscle cells were treated with insulin with or without palmitate for various time periods. Lipids (ceramides and TAG) were isolated and gene expression of multiple biosynthetic enzymes were quantified. Additionally, adult male mice received daily insulin injections for 14 days, followed by muscle ceramide analysis. *Results*. In muscle cells, insulin elicited an increase in ceramides comparable to palmitate alone. This is likely partly due to an insulin-induced increase in expression of multiple enzymes, particularly SPT2, which, when knocked down, prevented the increase in ceramides. In mice, 14 days of insulin injection resulted in increased soleus ceramides, but not TAG. However, insulin injections did significantly increase hepatic TAG compared with vehicle-injected animals. *Conclusions*. This study suggests that insulin elicits an anabolic effect on sphingolipid metabolism in skeletal muscle, resulting in increased ceramide accumulation. These findings reveal a potential mechanism of the deleterious consequences of the hyperinsulinemia that accompanies insulin resistance and suggest a possible novel therapeutic target to mitigate its effects.

1. Introduction

Diabetes mellitus is diagnosed on the basis of elevated blood glucose, despite significant changes in other blood markers, such as lipids, lactate, or insulin [1, 2]. The glucose emphasis is so strong that it provided the rationale for the disease name, which is understandable from a historic perspective, given that the main observable symptoms of the disease are indeed a consequence of glucose excess, including polyuria and its oft-noted sweetness [3]. However, while hyperglycemia represents the essential disorder of untreated type 1 diabetes mellitus, modern research reveals that many adverse consequences of type 2 diabetes mellitus are not a function of hyperglycemia, but rather of lipid and insulin excess [1, 4, 5].

The initial challenge to the long-embraced glucose-centric view of diabetes was issued by Denis McGarry [4] and others since [1, 6], when he posited that hyperinsulinemia and hyperlipidemia may be the more likely pathophysiological mediators of type 2 diabetes. Despite being widely prescribed in type 2 diabetes, insulin administration correlates with higher mortality rates [6, 7], and hyperinsulinemia is a factor in multiple diseases, including hypertension [8], fatty-liver disease [9, 10], PCOS [11], Alzheimer's disease [12], and more [13–15]. Though the lipid-centric aspect of McGarry's paradigm focused on triacylglycerol and free fatty acids, the sphingolipid ceramide may be the more relevant lipid regarding the deleterious consequences of type 2 diabetes and/or hyperlipidemia [16]. Indeed, increased intracellular ceramide is associated with similar diseases as hyperinsulinemia [10, 17–19].

Ceramide is an established mediator of insulin resistance in response to multiple insults [20, 21] and in diverse tissues [20, 22]. Additionally, muscle ceramide accumulation disrupts muscle metabolic function, altering mitochondrial structure, increasing ROS generation, and reducing mitochondrial respiration [23–25]. However, the role of insulin as an inducer of ceramide biosynthesis has never been explored. Not only would such a pathway identify a mechanism of negative feedback, wherein excessive insulin

signaling downgrades its own signal, but also it would fit the general lipid-anabolic actions of insulin, even in skeletal muscle [26, 27]. Thus, the purpose of these studies was to explore the novel role of insulin as an inducer of ceramide biosynthesis in skeletal muscle.

2. Materials and Methods

2.1. Animals. Sixteen-week-old male C57Bl/6 mice were separated into one of two groups at 12 weeks to receive daily saline or insulin (0.75 U/kg/BW) injections for 14 days with free access to water and chow. C57Bl/6 mice are commonly used as an ideal rodent model of exploring lifestyle risks associated with diabetes and obesity that mimic human responses [28]. Studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the IACUC (Institutional Animal Care and Use Committee) at Brigham Young University.

2.2. Cell Culture. C2C12 murine myoblast cells were maintained in DMEM (Dulbecco's modified Eagle's medium) plus 10% FBS (Invitrogen). For differentiation into myotubes, C2C12 myoblasts were grown to confluency and the medium was replaced with DMEM plus 10% horse serum (Invitrogen). Myotubes were used for experiments on day 4 of differentiation. For fatty acid treatment, palmitic acid (Sigma-Aldrich, catalogue number P5585) was dissolved in EtOH (ethanol) and diluted to the desired concentration in DMEM containing 2% (*w*/*v*) BSA (Sigma-Aldrich, A9576) and added to the cell culture at 0.5 mM, which is a physiological postprandial fatty acid concentration [29]. For *sptlc2* (serine palmitoyltransferase, long chain base subunit 2, also known as SPT2) knockdown, procedures were followed according to the manufacturer's instructions (Santa Cruz Biotechnology, sc-77377).

2.3. Lipid Isolation and Analysis. For isolation of lipids, pellets were resuspended in 900 μL of ice-cold chloroform/methanol (1 : 2), incubated for 15 min on ice, and then briefly vortex-mixed. Separation of aqueous and organic phases required addition of 400 μL of ice-cold water and 300 μL of ice-cold chloroform. The organic phase was collected into a fresh vial, and lipids were dried in a vacuum centrifuge (Eppendorf Concentrator Plus). Lipids were characterized and quantified using a shotgun lipidomics technique on a Thermo Scientific LTQ Orbitrap XL mass spectrometer. Evaporated lipid samples were resuspended in a 2 : 1 chloroform/methanol Folch solution (200 μL). The resuspended lipids were then combined with a modified 2 : 1 : 1.25 chloroform/methanol/propan-2-ol Bligh and Dyer solution (800 μL) with 15 mM ammonium acetate acting as an ionizing adduct. A 1.74 μM phosphatidylethanolamine internal standard (1 μL) was spiked into each sample for mass calibration and characterization data alignment. Samples were analyzed using a 2.5 min mass-window-scanning method in positive-ion mode at a resolution of 60000 (fwhm (full width at half maximum) at 400 m/z) for all primary MS^1 scans. MS^2 (tandem MS) fragmentation data were also collected and manually verified for each mass window to give additional confidence to the correct identification of abundant lipid species. Three technical replicate mass spectrometer runs were performed on each sample. Samples were injected at 10 μL/min using a direct-inject ESI soft-ionization spray head from a Hamilton GASTIGHT glass syringe. The spray voltage and capillary temperature were maintained at 5.0 kV and 275°C, respectively. Each technical replicate was run in random in order to reduce systematic bias. Data were analyzed using in-house-developed peak summarization, recalibration, and lipid identification software using lipid database information from the Lipid MAPS (LIPID Metabolites and Pathways Strategy) Lipidomics Gateway database [30]. To ensure high-confidence identifications, an intensity threshold estimated to be 5% above the instrumental static signal was implemented. Lipid identities were only assigned when significantly observable peaks were identified in at least two of the three technical replicate runs. Nonzero lipid quantities were averaged from the replicate runs. The lipid species identified across different ionization states or with adducts were totaled together. Quantification was completed by normalizing total ion counts to the relative abundance of the internal standard that was spiked into each sample.

2.4. Protein Analysis. Tissue and cell extracts lysed and protein content was determined using a BCA protein assay (Pierce) and the sample volumes were adjusted so that precisely 50 μg of protein was loaded into each lane. After the addition of sample buffer, samples were resolved by SDS/PAGE (10% gel), transferred onto nitrocellulose membranes, and immunoblotted using methods described previously [31]. After incubation with primary antibody, blots were incubated with an HRP- (horseradish peroxidase-) conjugated secondary antibody. HRP activity was assessed with ECL solution (Thermo Scientific) and exposed to film. The antibodies used were anti-SPT2 (Abcam; ab23696) and anti-rabbit IgG (Cell Signaling Technology, 7074S).

2.5. Real-Time qPCR. Total RNA was extracted and purified from tissues using TRIzol (Invitrogen) according to the manufacturer's recommendations. cDNA was synthesized from mRNA via reverse transcription—PCR using a commercial cDNA synthesis kit with oligo(dT) primers (iScript Select cDNA Synthesis, Bio-Rad Laboratories). Quantitative real-time PCR was performed with Evagreen Ssofast (Bio-Rad Laboratories) using a Bio-Rad Laboratories CFX Connect Real-Time PCR Detection system. Primer sequences were 5′-ACAGGATGCAGAAGGAGATTAC and 5′-CACAGAGTACTTGCGCTCAGGA as the forward and reverse primers, respectively, for *actb* (actin), 5′-TACTCAGAGACCTCCAGCTG and 5′-CACCAGGGATATGCTGTCATC for *sptlc1* (SPT1), 5′-GGAGATGCTGAAGCGGAAC and 5′-GTATGAGCTGCTGACAGGCA for *sptlc2* (SPT2), 5′-CACCGGTACCTCGGAGCGGA and 5′-GTTTGGGATTGATGAACAGGGGT for *des1* (Des1), 5′-CTGTTCTACTTGGCCTGTTG and 5′-TCATGCAGGAAGAACACGAG

for *lass1* (Cers1), 5′-CTCCAACGCTCACGAAATTC and 5′-ATGCAGACAGAAGATGAGTG for *lass 5* (Cers5), 5′-GTTCGGAGCATTCAACGCTG and 5′-CTGAGTCGTGAAGACAGAGG for *lass6* (Cers6), 5′-CTCGCTTGTCGTCTGCCT and 5′-TTGGCCCAGAACTCCTGTAG for FAS, and 5′-GTGCACAAGTGGTGCATCAG and 5′-CAGTGGGATCTGAGCCATCA for DGAT1. β-Actin reactions were performed side by side with every sample analyzed. Changes in the mRNA level of each gene for each treatment were normalized to that of the β-actin control mRNA according to Pfaffl [32].

3. Results

3.1. Insulin Increases Ceramide Biosynthesis in Muscle Cells. The primary observation of this report is that insulin treatment increases ceramides in murine myotubes. When treated with insulin (50 nM) for 16 h, ceramide levels increased roughly twofold (Figure 1). When assessing the insulin effects on gene expression, insulin increased the expression of genes encoding ceramide biosynthetic proteins SPT1 and 2, DES1 (Figure 2(a)), and FAS, involved in triacylglycerol synthesis (Figure 2(a)). Interestingly, the effects of insulin were comparable and even greater than palmitic acid (PA) treatment [20]. Moreover, the combined effects of both insulin and PA elicited an additive response with SPT2, resulting in a roughly 75% increase. In probing SPT2 gene expression further, we found that expression was significantly increased at 1 h of insulin treatment and peaked at 4 h (Figure 2(b)). A somewhat distinct trend was observed with actual ceramides, which peaked at 8 h (Figure 2(c)) and remained comparably elevated at 16 h of treatment.

3.2. SPT2 Is an Important Regulator of Insulin-Induced Ceramide Accrual. SPT2 was knocked down in murine myotubes via siRNA. To confirm the functional effects of the knockdown, SPT2 transcript level was measured in control (scramble) and SPT2 siRNA-treated cells with BSA and palmitic acid (PA) incubation, which is a well-established substrate and inducer of ceramide biosynthesis [33]. Transcript levels of SPT2 were reduced compared to control cells in both BSA- and PA-treated conditions (Figure 3). Moreover, SPTKD cells failed to respond to PA treatment (Figure 3). Further, while ceramides levels were not significantly different in control and SPT2 KD cells in control conditions (CON), insulin (INS), PA, and insulin with PA (INS + PA) elicited a significant increase in ceramides in the control cells, but not the SPT2 KD cells (Figure 4(a)). Moreover, combination of insulin and PA (INS + PA) had a greater effect than INS and PA alone (Figure 4(a)). Interestingly, the SPT2 KD cells had higher TAG levels than control cells and experienced a significant increase in TAG levels with PA and INS + PA (Figure 4(b)).

3.3. Insulin Injections Increase Muscle Ceramides in Mice. Mice were injected with either insulin (0.75 U/kg) or PBS daily at the beginning of the light cycle for 14 days. Mice were allowed free access to food and water throughout the study period. At the conclusion of the study period, insulin-injected animals gained significantly greater body weight (Figure 5(a)), despite similar food consumption (Figure 5(b)). Gene expression levels in the muscle from insulin-injected animals tended to follow the trends observed in muscle cells. Specifically, levels of SPT1, SPT2, and FAS were elevated, but not DES1 and DGAT1 (Figure 6). Lastly, insulin injection had a particularly potent effect on muscle ceramide levels, but, as a comparison, not liver ceramides (Figure 7(a)). In contrast to ceramides, TAG levels in the soleus were not significantly elevated with insulin (Figure 7(b)). However, insulin injections did cause increased liver TAG (Figure 7(b)).

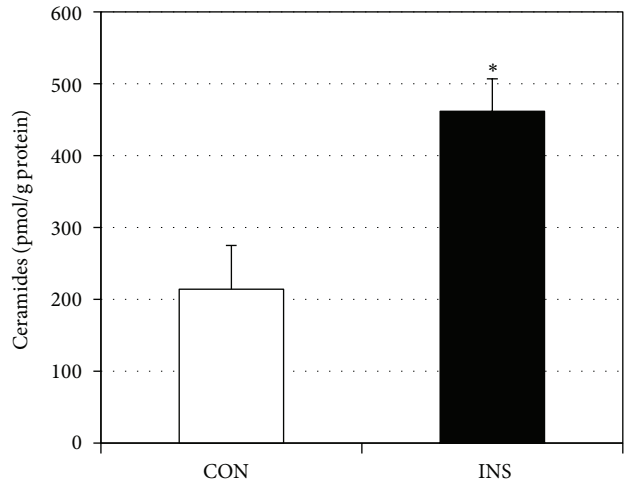

Figure 1: Murine myotubes were treated with vehicle (CON) or insulin (INS, 50 nM) for 16 h. Following treatment, ceramides were isolated and quantified. $^{*}P < 0.05$.

4. Discussion

This study evaluated the direct effect of insulin on skeletal muscle ceramide metabolism. The primary observation revealed that skeletal muscle cells respond to insulin treatments with a significant increase in ceramide, a finding further confirmed with increased ceramide in skeletal muscle from mice receiving daily insulin injections. This is, at least partly, a result of insulin-induced alterations in expression of enzymes involved in lipid handling (i.e., glycerolipid and sphingolipid). In particular, insulin increased expression of two isoforms of the initial and rate-limiting step in de novo ceramide biosynthesis (e.g., SPT1 and 2), as well as the final step, involving desaturation of the sphinganine backbone of dihydroceramide (DES1). Also, insulin increased FAS expression in both muscle cell cultures and whole muscle.

Our finding of increased ceramide accrual with insulin adds a layer of complexity to the abundant observations of increased skeletal muscle ceramide in insulin resistant-hyperinsulinemic states [34–36]. Whereas the basic assumption is that ceramide exacerbates insulin resistance and this is well supported [33, 37–40], the present findings suggest that

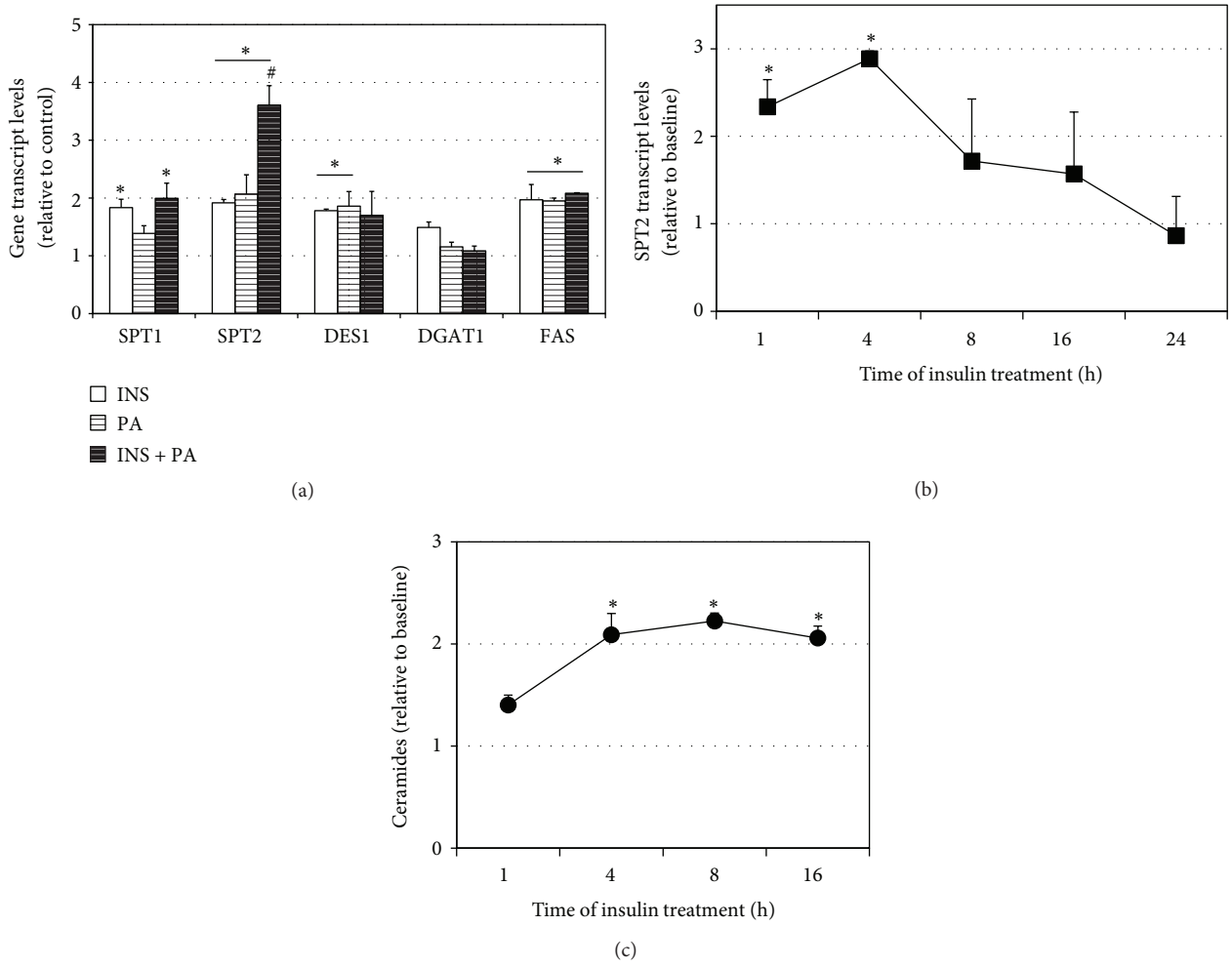

Figure 2: Murine myotubes were treated with insulin (50 nM) with or without palmitic acid (PA, 0.5 mM) for 16 h. (a) Following treatment, expressions of multiple enzymes involved with ceramide (SPT1, SPT2, and DES1) and TAG (DGAT1, FAS) metabolism were quantified. $^{*}P < 0.05$, treatment versus control. $^{\#}P < 0.05$, INS + PA versus other treatments. (b) The time course of insulin treatment (50 nM) revealed that SPT2 gene expression was significantly increased at 1 h and peaked at 4 h. $^{*}P < 0.05$, treatment versus control. (c) The time course of insulin treatment (50 nM) revealed that ceramides were significantly increased at 4 h and sustained through 16 h. $^{*}P < 0.05$, treatment versus control.

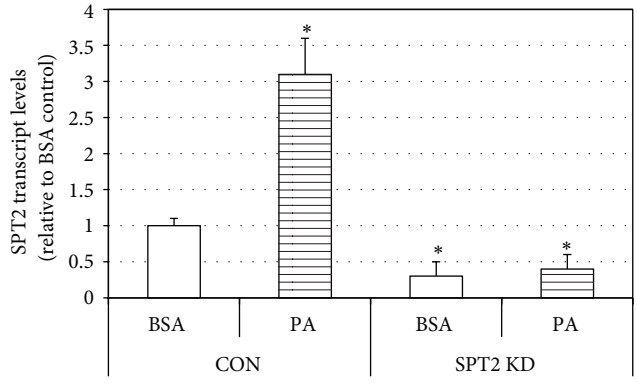

Figure 3: To confirm efficacy of SPT2 knockdown in murine myotubes, cells were treated with palmitic acid (PA, 0.5 mM). $^{*}P < 0.05$, PA versus BSA.

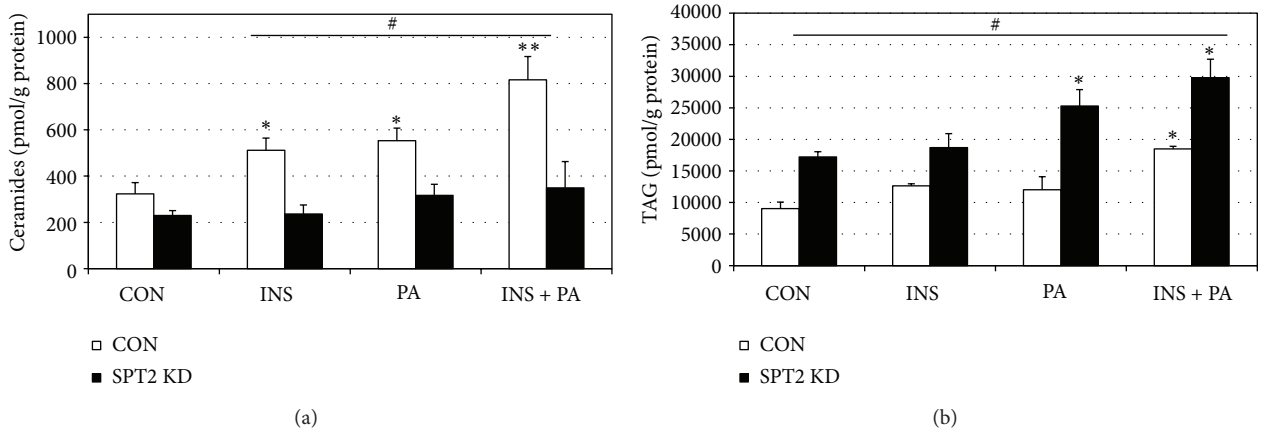

Figure 4: Following SPT2 knockdown, myotubes were treated with insulin (INS, 50 nM), palmitic acid (PA, 0.5 mM), or both (INS + PA). (a) Ceramides were significantly comparably elevated with INS and PA treatments, but not with SPT2 KD ($^{*}P < 0.05$, treatment versus control). Both treatments together (INS + PA) resulted in an additive increase in ceramides ($^{**}P < 0.05$, INS + PA versus INS and PA). SPT2 KD prevented treatment-induced increases in ceramide ($^{\#}P < 0.05$, CON (scramble) versus SPT2 KD). (b) TAG levels were elevated in PA and INS + PA treatments ($^{*}P < 0.05$, treatment versus control). SPT2 KD increased TAG in every condition ($^{\#}P < 0.05$, SPT2 versus CON (scramble)).

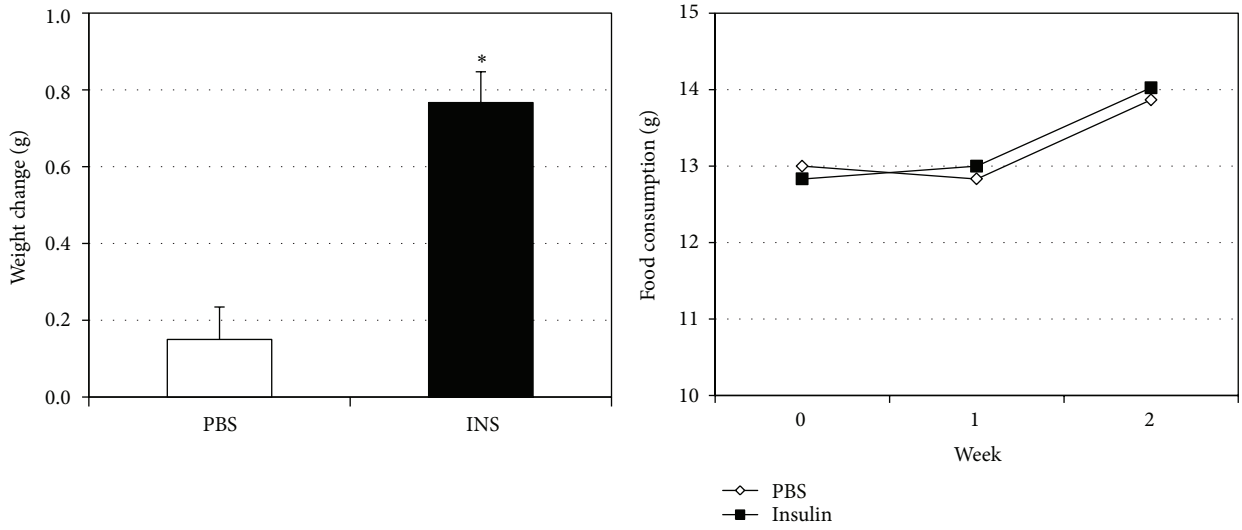

Figure 5: Adult male mice (12 weeks) were divided into two groups to receive insulin (INS, 50 nM) or vehicle (PBS) injections every morning for 14 d. (a) Body weights were measured before and following treatment period. (b) Weekly food consumption was determined for the week prior to injection (week 0) and for each of the two following weeks of treatment. $^{*}P < 0.05$, INS versus PBS.

ceramide accumulation may be both a cause and consequence of hyperinsulinemia. Given ceramide's actions as an insulin antagonist, it is noteworthy that insulin increases ceramide accumulation in muscle. This phenomenon may indicate a degree of insulin self-regulation, wherein insulin downregulates its signaling when in excess, such as with the hyperinsulinemia accompanying insulin-resistant states. Interestingly, recent work by Zabielski et al. [41] established that insulin deprivation increased muscle ceramides by ~50%. Specifically, after inducing type 1 diabetes mellitus through streptozotocin treatment, Zabielski et al. [41] found that insulin deprivation increased quadriceps ceramide content. Thus, combined with our results, these reports collectively suggest that both lack of and excess of insulin increase muscle ceramide accrual.

Our findings of a significant increase in TAG with SPT2 KD are relevant given the expanding appreciation of TAG accrual in muscle being benign with regard to insulin resistance. Known as the athlete's paradox, this phenomenon was first observed by Goodpaster et al. [42]. Since that time,

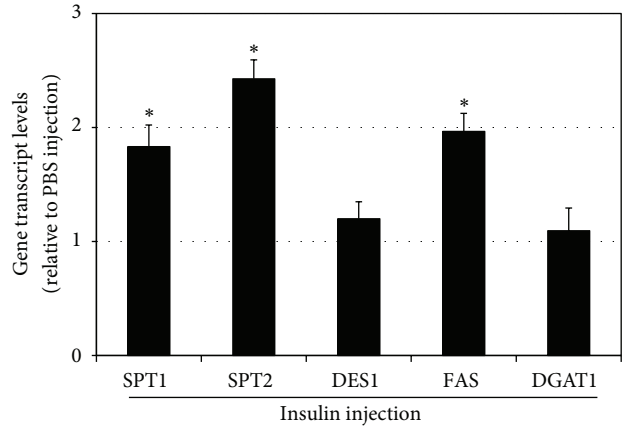

Figure 6: Adult male mice (12 weeks) were divided into two groups to receive insulin (INS, 50 nM) or vehicle (PBS) injections every morning for 14 d. Soleus levels of genes of multiple enzymes involved with ceramide (SPT1, SPT2, and DES1) and TAG (DGAT1 and FAS) metabolism were quantified. $^{*}P < 0.05$, INS versus PBS.

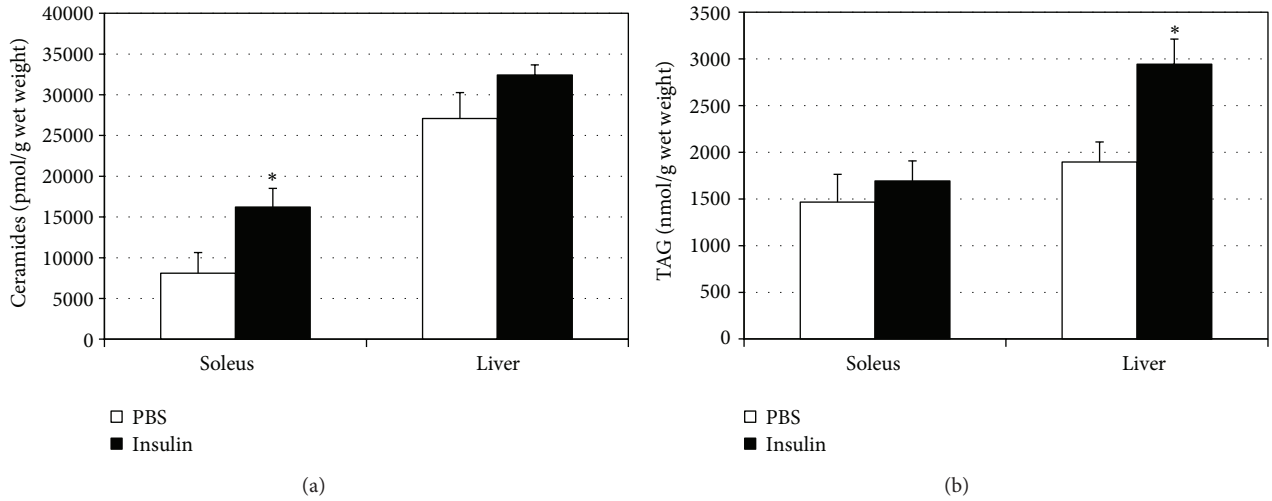

Figure 7: Following treatment period, soleus and liver were dissected from adult male mice receiving insulin (INS, 50 nM) or vehicle (PBS) injections every morning for 14 d. Ceramides (a) and TAG (b) were quantified from both tissues. $^{*}P < 0.05$, insulin versus PBS.

shuttling lipid into the glycerolipid pathway in muscle is now considered a protective effect to insulin signaling. Liu et al. [43] found that mice that accumulate more TAG levels in muscle are protected against diet-induced insulin resistance. Importantly, this was associated with reduced ceramide content. We believe that a similar effect occurs with SPT2 KD; rather than shuttling carbons into sphingolipid biosynthesis with the various treatments (Figure 4), which is blunted due to knockdown of the rate-limiting step, the cell is increasing glycerolipid synthesis, evident by the increased TAG levels. Collectively, these observations suggest an insulin-sensitizing effect of ceramide inhibition and subsequent TAG synthesis.

Throughout these studies, palmitate was used as a positive control. Palmitate has long been known to increase ceramide accrual [33, 37], and it is noteworthy that insulin had a comparable effect on muscle ceramides as palmitate. Palmitate is important for ceramide biosynthesis for two reasons—as a substrate for ceramide creation (via SPT) and as an activator of ceramide biosynthetic pathways [20]. Our observation of an additive increase in ceramides with insulin and palmitate together in muscle cells is noteworthy because it reflects an environment that typifies insulin resistance—elevated insulin, increased circulating fatty acids, and increased muscle ceramides [34, 36, 44, 45]. The knowledge that both insulin and fatty acids increase ceramide highlights the potential importance of ceramide as a mediator of complications associated with elevations in both factors.

Multiple studies indicate that chronic exposure to insulin leads to insulin resistance [6], with dramatic increases in mortality from multiple diseases [46]. While the discussion is particularly relevant to type 2 diabetes, the ramifications similarly apply to type 1 diabetes. Deckert et al. [47] observed that type 1 diabetics that require less insulin have higher rates of survival. Altogether, these observations, in conjunction with

our results, suggest the need for careful consideration when treating type 2 diabetes mellitus with insulin, especially when insulin levels are already elevated [6]. Whether ceramide is the primary regulator of reduced insulin sensitivity with chronic insulin is unknown.

While insulin resistance is known as a consequence of obesity [48], insulin is also critical in the expansion of adipose tissue that typifies obesity. Indeed, we observed a significant increase in body weight in the insulin-injected animals compared with the saline-injected mice (Figure 5(a)), despite no significant difference in food consumption. While the treatment period is relatively short, these findings nonetheless corroborate a substantial body of evidence showing the uniquely potent fattening effect of insulin [49–53], regardless of calories consumed [54, 55].

Insulin therapy is a common practice for treating type 2 diabetes mellitus. While this is undeniably effective at controlling blood glucose and mitigating the likelihood of hyperglycemia, the patient can suffer from the complications of hyperinsulinemia, including increased mortality [7] and weight gain [56]. Two thoughts arise from these observations. First, ideally the disease is detected in the early stage known as "prediabetes" or insulin resistance. However, this stage is typified by patients experiencing hyperinsulinemia, but not necessarily hyperglycemia. In other words, the pancreas is producing sufficient, if increased, insulin to maintain normoglycemia. Because blood glucose is typically used as the diagnostic marker of diabetes mellitus, but not insulin, many of these individuals remain undiagnosed [57]. Thus, insulin should be measured in clinical situations to allow the earlier detection of diabetes mellitus. Second, increasing efforts should be focused on lifestyle to control glucose and insulin, particularly diet. Carbohydrate-restricted diets, even in the absence of calorie restriction, are highly effective at reducing blood glucose and type 2 diabetics are often able to reduce the dose of or stop taking diabetic medications altogether [58].

5. Conclusions

In conclusion, our findings of increased ceramide biosynthesis with insulin provide a possible mechanism to partly explain the substantial evidence linking hyperinsulinemic conditions (e.g., insulin resistance and type 2 diabetes) to multiple disease states, especially vascular complications. For example, insulin resistance is associated with a significant increase in atherosclerosis [59] and ceramide increases atherosclerotic lesion development [60]. Similarly, insulin resistance is a common causal factor in hypertension [61], and vascular ceramide accrual compromises vasodilation [62, 63], increasing blood pressure. Thus, anticeramide therapies may prove to be a viable therapy for combating certain insulin-induced disorders, which is the focus of ongoing efforts.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Vomiting and Dysphagia Predict Delayed Gastric Emptying in Diabetic and Nondiabetic Subjects

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Background. Gastroparesis is a heterogeneous disorder most often idiopathic, diabetic, or postsurgical in nature. The demographic and clinical predictors of gastroparesis in Israeli patients are poorly defined. *Methods.* During the study period we identified all adult patients who were referred to gastric emptying scintigraphy (GES) for the evaluation of dyspeptic symptoms. Of those, 193 patients who were referred to GES from our institution were retrospectively identified (76 (39%) males, mean age 60.2 ± 15.6 years). Subjects were grouped according to gastric half-emptying times (gastric $T_{1/2}$). Demographic and clinical data were extracted from electronic medical records or by a phone interview. *Key Results.* Gastric emptying half-times were normal (gastric $T_{1/2}$ 0–99 min) in 101 patients, abnormal (gastric $T_{1/2}$ 100–299 min) in 67 patients, and grossly abnormal (gastric $T_{1/2} \geq 300$ min) in 25 patients. Vomiting and dysphagia, but neither early satiety nor bloating, correlated with delayed gastric emptying. Diabetes was associated with grossly abnormal gastric $T_{1/2}$. Idiopathic gastroparesis was associated with a younger age at GES. No correlation was observed between gastric $T_{1/2}$ values and gender, smoking, *H. pylori* infection, HBA1C, or microvascular complication of diabetes. *Conclusions Inferences.* Vomiting and dysphagia are predictive of delayed gastric emptying in both diabetic and nondiabetic subjects. Diabetes is associated with more severe gastroparesis.

1. Introduction

Gastroparesis is a condition of impaired gastric emptying without evidence of gastric outlet obstruction. This disorder is characterized by a poor quality of life and nutritional deficits and is associated with symptoms such as nausea, vomiting, postprandial fullness, early satiety, and bloating [1–3]. Gastroparesis is a heterogeneous disorder most often idiopathic, diabetic, or postsurgical in nature, affecting up to 1.8% of the population. Diabetic gastroparesis (DG) affects patients with long-standing diabetes mellitus usually complicated by retinopathy, neuropathy, and nephropathy [1]. The clinical and histopathological features of idiopathic gastroparesis are variable and poorly defined. For example, *H. pylori* infection has been reported to increase [4], decrease [5, 6], or not influence [7–10] the likelihood of delayed gastric emptying. Idiopathic gastroparesis has been described predominantly in young female patients with low-normal body mass [11]. The predictive value of dyspeptic symptoms is also the subject of ongoing study [11–17]. Recently, a Gastroparesis Cardinal Symptom Index (GCSI) has been developed as a valid tool for symptom stratification and for the evaluation of treatment response [3, 18]. Nevertheless, precise clinical correlates of gastroparesis remain elusive. In the first study of its sort from our geographical region, we attempt to further define the predictors of delayed gastric emptying in patients undergoing gastric emptying scintigraphy (GES) at our tertiary referral center.

2. Methods

2.1. Patients. This single-center study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice (GCP) and was approved by the Human Subjects Protection Program of the Rabin Medical Center (RMC). Dyspeptic patients undergoing GES at our institution between January 2003 and December 2009 were retrospectively identified using an established computerized chart. Only patients examined at the gastroenterology outpatient clinic and referred to GES by a gastroenterologist were included. Similarly, only subjects who had undergone upper gastrointestinal endoscopy within 1 year of GES were included. This was to ensure that no patients had gastric outlet obstruction as a cause of their symptoms. The following cases were excluded: patients with established gastroparesis undergoing follow-up GES, cases lacking *H. pylori* testing by histology, rapid urease test, or C-13 urea breath test within 3 months of GES, gastric outlet obstruction, active malignancy, pregnancy, age below 18 years, and incomplete medical records.

2.2. Data Collection. The following parameters were obtained from the patients' electronic record: age, sex, symptoms (dysphagia, early satiety, nausea, vomiting, bloating, abdominal pain, heartburn, and regurgitation), smoking, other active health problems including ischemic heart disease, gastroesophageal reflux disease, rheumatologic disease including scleroderma, endocrine disease including diabetes mellitus (noting microvascular complications), and thyroid disease, current medications including antireflux, opioid analgesics, and promotility agents. The electronic records retrieved included admission data, clinic visits, billing claims data, ICD-9 diagnoses registered in the centralized database, and pharmacy claims. All data were obtained by two independent reviewers (Doron Boltin and Ibrahim Zvidi). Missing parameters were obtained by a phone interview (Ibrahim Zvidi).

2.3. Gastric Empting Scintigraphy. Following a 14-hour fast patients received a standard 250 kca meal consisting of an egg fried in 5 g margarine, 2 slices of white bread, and 200 mL of water (15 g proteins, 26 g carbohydrates, and 9 g fat). Isotope labeling was performed by adding 1 mCi of ^{99m}Tc-sulphur colloid to the egg white. Fixation of the tracer to the solid phase (necessary for measuring gastric emptying of solids) was accomplished by dissolving the isotopes inside the egg and solidifying the egg. Sequential-conjugated anterior-posterior view scintigrams of the epigastric area were acquired in a sitting position on a dual head gamma camera (Milennium VG and Infinia, GE, Buckinghamshire, UK, and E.cam, Siemens, Buckinghamshire, UK) at 30, 60, and 120 minutes following ingestion of the standardized test meal. After 2008, delayed scans were performed at 180 and 240 minutes in accordance with guidelines published at that time [19].

2.4. Data Analysis. To analyze the scintigraphy results the gastric region of interest (ROI) was manually drawn around the stomach on the frames at the beginning of the dynamic scan. A time-activity curve was generated from the ROI and was corrected for radioisotope decay. A linear fit of the time activity curve was used to calculate the gastric emptying half-time (gastric $T_{1/2}$). A gastric $T_{1/2}$ greater than 100 minutes was considered abnormal. Gastric retention, a recently introduced method to define gastric emptying rate, is considered by most authors as a better method to assess gastric emptying rate (GER). Gastric retention greater than 10% after 4 hours is considered abnormal [19]. In this study, during GES we were able to collect both gastric $T_{1/2}$ and gastric retention values. However, assessment of gastric retention of tracer was feasible only after 2008 and for this reason we used gastric $T_{1/2}$ for statistical analyses.

2.5. Statistical Analysis. Due to the presence of incalculable $T_{1/2}$ values for gastric emptying (excessively prolonged) data was categorized into 3 groups as opposed to a continuum. Univariate analysis was performed for each of the acquired variables after sorting patients according to their *H. pylori* status and gastric emptying. Individual and cumulative scores were expressed as mean ± 1SD. Student's t test was used for continuous variables including age. Categorical variables including sex, clinical diagnosis, *H. pylori* infection, and $T_{1/2}$ were analyzed with Pearson's χ^2 test and Fischer exact test. Mann-Whitney U test was used for symptom scores. As dependent variables we considered either individual symptom scores or the cumulative scores of symptoms. All possible cutoff values of the scores for these dichotomous variables were considered in the analysis. P values of 0.10 and 0.15 were chosen as cutoff points to enter and exit the stepwise procedure. Odds ratios (OR) with 95% confidence intervals (CI) were computed by means of χ^2 analysis only for the independent variables that entered the model. A cutoff value of ≥40 yr was chosen for age. Statistical evaluation was performed using the software package SPSS 21.0 (SPSS Inc., Chicago, IL).

3. Results

During the study period, 420 dyspeptic patients underwent GES at our institution of whom 193 (46%) were eligible for inclusion in the study (76 (39%) males, mean age 60.15±15.61 years). Patient characteristics are summarized in Table 1. There was a preponderance of females in all groups which did not correlate with $T_{1/2}$ values. Relevant past medical history included type II diabetes mellitus in 79 patients (40.9%), scleroderma in 14 patients (7.3%), and previous esophageal/gastric surgery in 19 patients (9.8%). Evidence of *H. pylori* infection was found in 42 patients (21.8%). The $T_{1/2}$ for gastric emptying was normal (0–99 minutes) in 101 patients (group 1), abnormal (100–299 minutes) in 67 patients (group 2), and excessive (≥300 minutes) in 25 patients (group 3). Significant associations between prolonged gastric emptying and patient characteristics are shown in Table 2. We found a significant association between prolonged gastric $T_{1/2}$ and presenting symptoms of dysphagia or vomiting and the use of antisecretory or promotility agents (Figure 1). This was true for both patients with diabetes and those without diabetes. Diabetes was associated with significantly

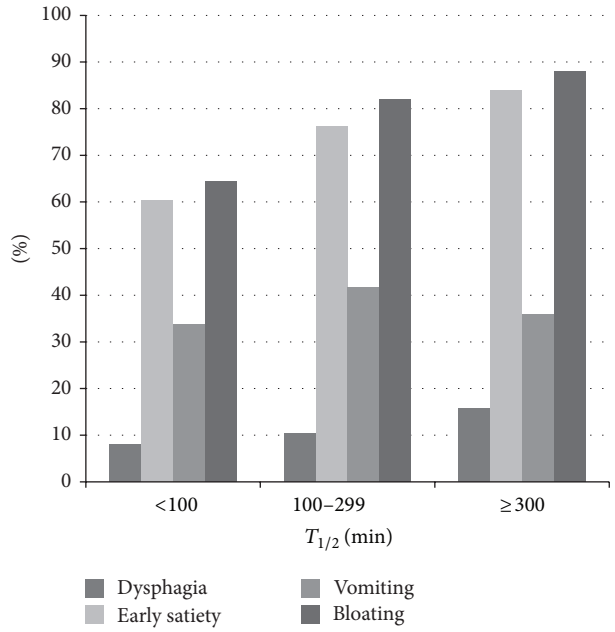

Figure 1: Prevalence of upper gastrointestinal tract symptoms in subjects undergoing gastric emptying scintigraphy.

more excessive results (group 3) compared to group 2 (OR 1.98 (0.77–5.00; $P = 0.03$)); however, when compared to patients with normal gastric emptying (group 1), diabetes was not more common (Figure 2). There was no significant correlation between gastric $T_{1/2}$ and patient age, gender, or *H. pylori* infection. Subgroup analysis demonstrated that patients with idiopathic gastroparesis were significantly younger than their counterparts with gastroparesis secondary to diabetes, surgery, or scleroderma; however, no difference in sex, *H. pylori* infection, or any other predictor was observed (Table 3).

4. Discussion

In the present study we describe the demographic and clinical predictors of gastroparesis in a cohort of consecutive dyspeptic patients referred for gastric scintigraphy at a single tertiary referral center. Our center is the largest hospital belonging to Clalit Health Services, the largest of five health care providers in the country with approximately 3.8 million members. This is the first such study emerging from Israel.

Of all the presenting symptoms in subjects referred for scintigraphy, only dysphagia and vomiting were independent predictors of a delayed gastric $T_{1/2}$. These findings concur with Ardila-Hani et al. [12] who found positive correlation between vomiting and anorexia and delayed gastric $T_{1/2}$. Interestingly, bloating was negatively correlated with gastric retention. Others have found that postprandial fullness, bloating, and pain correlate with delayed gastric emptying, but not vomiting or dysphagia [15, 20]. Ron et al. found that early satiety was the only patient-reported symptom associated with delayed gastric emptying, as assessed by breath-test [21]. Prospective studies using validated scoring systems have correlated protean symptoms with gastric retention; however, a wide variability exists [11–13, 16]. In fact, delayed gastric emptying rate (GER) is not always associated with symptoms. Indeed, gastric $T_{1/2}$ is not a very specific marker of gastric dysmotility, and it has been found to be surprisingly normal in some dyspeptic patients with long-standing diabetes mellitus [21].

Dysphagia, as a predictor of gastric emptying has not been reported by other groups, is not a classic manifestation of gastroparesis and indeed is not a component of the GCSI. Our computerized charts allowed for reliable reporting of dysphagia. Nevertheless, this finding may be biased by an overrepresentation of scleroderma and upper GI surgery cases (in whom dysphagia was reported in 21%, compared to 11% in diabetic and idiopathic cases) or an overrepresentation of patients with GERD (in whom dysphagia was noted

Table 1: Patient characteristics.

	N (%)
N	193 (100)
Age y (mean (SD))	60.15 (15.61)
Male	76 (39.38)
H. pylori	44 (22.80)
Smoking	20 (10.36)
Symptoms	
Dysphagia	25 (12.95)
Early satiety	133 (68.91)
Vomiting	71 (36.79)
Bloating	142 (73.58)
Diabetes	79 (40.93)
HBA1C[§] % (mean (SD))	7.70 (1.87)
Neuropathy	34 (43.04)
Retinopathy	41 (51.90)
nephropathy	25 (31.65)
Antidiabetic medication[¥]	76 (96.20)
Cardiovascular disease	139 (72.02)
Hypertension	105 (54.40)
Dyslipidemia	132 (68.39)
Ischemic heart disease	43 (22.28)
Peripheral vascular disease	22 (11.40)
Scleroderma	14 (7.25)
Gastroesophageal surgery	18 (9.32)
GERD	94 (48.70)
Medications	
Oral hypoglycemic	51 (26.42)
Insulin	34 (17.62)
Antiaggregant[¶]	96 (49.74)
Antihypertensive	108 (55.96)
Antisecretory[#]	147 (76.17)
Narcotic	7 (3.63)
Metoclopramide /domperidone	57 (29.53)

[§]Data missing for 5 subjects; [¥]insulin, oral hypoglycemic agents, or both; [¶]aspirin, clopidogrel, or both; [#]H2-receptor antagonist (10 cases), proton pump inhibitor (126 cases), or both (11 cases).

Table 2: Independent predictors of prolonged gastric emptying.

	OR (95% CI)			P
	Group 3/group 1*	Group 2/group 1*	Group 3/group 2*	
Dysphagia	3.44 (1.10, 10.78)	2.09 (1.05, 4.16)	1.65 (0.49, 6.00)	0.021
Vomiting	4.06 (1.14, 14.51)	2.54 (1.20, 5.35)	1.60 (0.41, 6.00)	0.008
Diabetes	0.83 (0.35, 2.01)	0.42 (0.22, 0.81)	1.98 (0.77, 5.00)	0.032
Proton pump inhibitor	0.94 (0.38, 2.35)	2.43 (1.15, 5.13)	0.39 (0.14, 1.00)	0.046
H2 antagonist	2.34 (0.78, 7.02)	0.35 (0.09, 1.28)	6.74 (1.54, 30.00)	0.002
Metoclopramide	2.43 (0.93, 6.32)	3.29 (1.64, 6.60)	0.74 (0.29, 2.00)	0.002

* Group 1: $T_{1/2}$ 0–99 minutes; group 2: $T_{1/2}$ 100–299 minutes; group 3: $T_{1/2} \geq 300$ minutes.

Table 3: Clinical associations of gastroparesis.

	Group 1¥	Group 2	Group 3	Total
Idiopathic *N* (%)	46 (49.5)	36 (38.7)	11 (11.8)	93 (100)
Male *N* (%)	20 (43.5)	13 (36.1)	3 (27.3)	36 (38.7)
Age mean (SD)	59.20 (17.7)	57 (19.5)	54.64 (17.8)	57.81 (18.3)
H. pyloriN (%)	11 (23.9)	8 (22.2)	3 (27.3)	22 (23.7)
Secondary§ *N* (%)	55 (55.0)	31 (31.0)	14 (14.0)	100 (100)
Male *N* (%)	25 (45.5)	11 (35.5)	4 (28.6)	40 (40.0)
Age mean (SD)	63.45 (11.3)	59.29 (13.9)	64.57 (12.1)	62.32 (12.3)*
H. pyloriN (%)	11 (20.0)	5 (16.2)	6 (42.9)	22 (22.0)
Diabetes *N* (%)	49 (62.0)	13 (16.5)	17 (21.5)	79 (100)
Male *N* (%)	20 (40.8)	5 (38.5)	6 (35.3)	31 (39.2)
Age mean (SD)	63.7 (11.2)	64.2 (9.6)	64.1 (11.2)	63.8 (10.8)*
H. pylori N (%)	10 (20.4)	1 (7.7)	6 (35.3)	17 (21.5)

* $P < 0.05$ (compared to idiopathic group).
§ Diabetes mellitus, scleroderma, and prior surgery.
¥ Group 1: $T_{1/2}$ 0–99 min; group 2: $T_{1/2}$ 100–299 min; group 3: $T_{1/2} \geq 300$ min.

in 13.8%, compared to 9.1% without GERD). So, too, our exclusion of patients without upper GI endoscopy within 12 months of GES may have led to the overrepresentation of alarm symptoms such as dysphagia.

We found no correlation between prolonged gastric emptying and *H. pylori* infection, either as a whole group or in the subset of patients with idiopathic gastroparesis. This is in keeping with several older studies which have discounted a specific link between *H. pylori* and idiopathic gastroparesis [22, 23]. Although a minority of studies have linked *H. pylori* infection to increased or decreased gastric emptying, these studies are limited by inaccurate definitions of dyspepsia, small case numbers, nonstandardized symptom questionnaires, and methodological flaws such as the use of low-calorie test meals [5, 6, 24].

Over 40% of subjects included in this study had comorbid type II diabetes mellitus. Diabetes is a well-described cause of gastroparesis (29%) and the incidence of gastroparesis is 4.5% and 1.0% in types I and II diabetes, respectively [25]. Diabetic gastroparesis may be related to autonomic neuropathy, enteric neuropathy, interstitial cells of Cajal dysfunction, acute hyperglycemia, incretin-based medications, and altered neuroendocrine function [25]. In this study, patients with a markedly abnormal gastric $T_{1/2}$ (≥300 mins) were almost twice as likely to have diabetes mellitus, when compared to subjects with lesser degrees of gastroparesis (100 mins $\leq T_{1/2} < 300$ mins). Interestingly, a similar relationship was not observed when compared to patients with normal gastric emptying ($T_{1/2} < 100$) (Table 2). This may reflect a referral bias to scintigraphy for diabetic patients with upper gastrointestinal symptoms, leading to an overrepresentation of diabetic subjects in group 1. Alternatively, this may be a reflection of relatively well-controlled diabetes in study population (mean HbA1C was 7.7%) or preemptive treatment with promotility agents. Subanalyses did not reveal any difference in gastric $T_{1/2}$ between diabetic subjects with or without microvascular complications.

Antisecretory medications were positively correlated with gastric retention. This is probably a confounding factor, simply representing a subset of patients with severe symptoms. Proton pump inhibitors and H2-receptor antagonists have no intrinsic effect on gastric emptying rate [26]. Patients receiving promotility agents had prolonged gastric emptying. This likely represents (inadequate) treatment in patients with a high pretest probability for gastroparesis. Suffice it to say, current recommendations call for stopping all medications

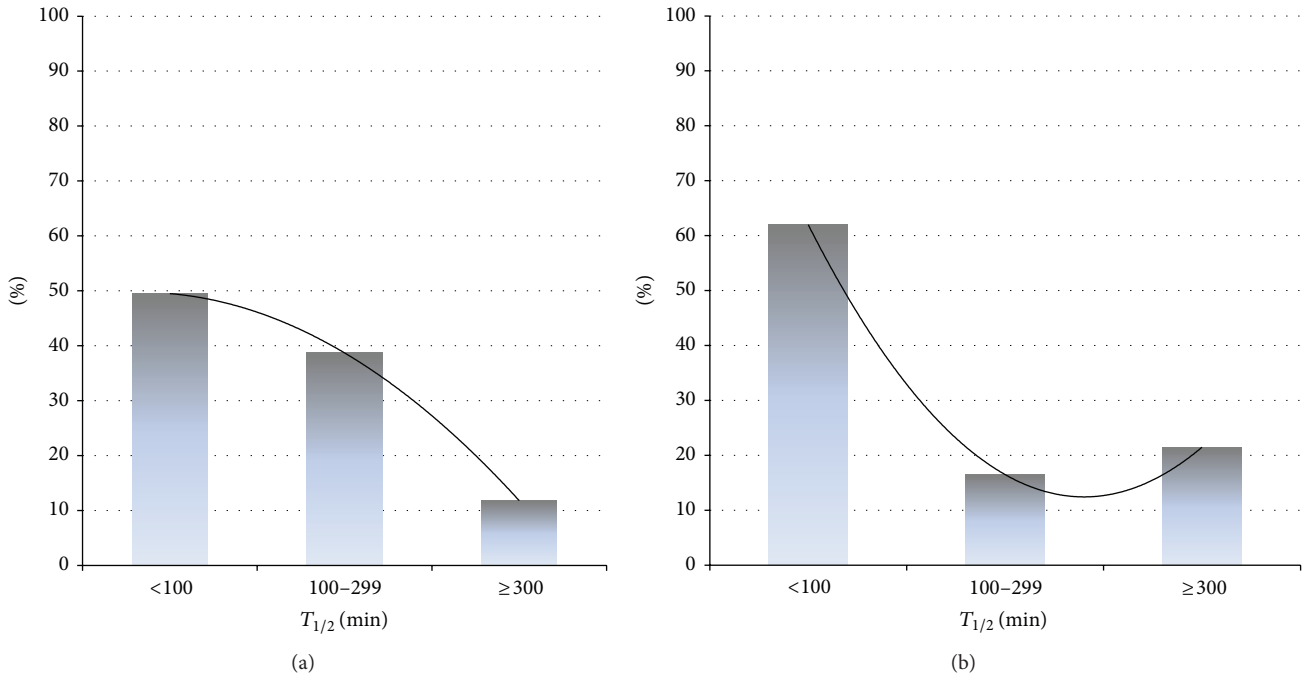

Figure 2: Gastric emptying in idiopathic and diabetic subjects. (a) Percentage of previously healthy (idiopathic) subjects. (b) Percentage of diabetic subjects with normal (<100 mins), mildly delayed (100–299 mins), and grossly delayed gastric emptying (≥300 mins).

known to affect gastric emptying for 48–72 hours; however, due to the retrospective nature of our study it is impossible to know if this was enforced.

Subgroup analysis revealed that patients with idiopathic gastroparesis were significantly younger than their counterparts with diabetes, previous surgery, or rheumatic disease. Two-thirds of subjects with idiopathic gastroparesis were female, compared to approximately half of subjects with normal gastric emptying. This is consistent with Parkman et al. who found that idiopathic gastroparesis occurs predominantly in young female patients with low-normal body mass [11, 27]. The underlying mechanism for this phenomenon is not fully understood and may be related to estrogen levels. Indeed, during the ovulatory period and pregnancy, peristalsis is decreased and constipation is commonly reported [28]. Parkman, however, described a cohort providing no comparison to other forms of gastroparesis or to subjects undergoing GES without gastroparesis.

Our study has several limitations, including the retrospective design and the absence of validated symptom questionnaires such as the GCSI. Nevertheless, every attempt was made to retrieve data on symptoms retrospectively in a thorough manner. Where missing, data was retrieved from phone interviews. All of the symptoms mentioned in Section 2 were included in the analysis and only vomiting and dysphagia were found to be independent predictors. No reliable data on body mass index (BMI) could be extracted from the electronic files. Another limitation is our inclusion of variables which may affect gastric emptying in unpredictable and unquantifiable manner. These potential confounders include GERD, prior surgery, scleroderma, and antisecretory medication. Data was partially obtained from scans performed prior to 2009 at which time a standardized test protocol (minimum 4-hour testing) was adopted [19]. For this reason extrapolated $T_{1/2}$ values were used to quantify gastric emptying as opposed to the directly measurable percentage of tracer retained. This in turn precluded regarding gastric emptying as a continuous variable, as $12T_{1/2}$ values were "infinite." Each of these factors may affect the reliability of the results. Finally, our cohort included 18 subjects with prior upper GI surgery. There is currently no data regarding what constitutes normal or abnormal gastric emptying in these patients.

In conclusion, this study identifies that vomiting and dysphagia but not bloating or early satiety are independent predictors of prolonged gastric emptying. Diabetes is associated with excessively prolonged gastric emptying. Idiopathic gastroparesis is a disease of younger women. Large well-designed prospective cohorts are needed to verify these findings.

Disclosure

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Doron Boltin prepared the paper. Doron Boltin, Yuval Nardi, and Mona Boaz performed the data analysis. Ibrahim Zvidi, Adam Steinmetz, and Hanna Bernstine performed the research. David Groshar and Yaron Niv provided essential equipment. Ram Dickman designed the study protocol and oversaw implementation at all stages.

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Review Article

Association between eNOS 4b/a Polymorphism and the Risk of Diabetic Retinopathy in Type 2 Diabetes Mellitus: A Meta-Analysis

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Many studies have assessed the association between eNOS-4b/a polymorphism and the risk of diabetic retinopathy (DR) among type 2 diabetic subjects. However, the results are inconsistent. In order to derive a more precise estimation of the association, a meta-analysis was conducted. Fifteen studies with 3, 183 cases and 3, 410 controls were enrolled by searching the databases of Pubmed, Embase, China National Knowledge Infrastructure (CNKI), and Chinese Wanfang Database. Summary odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. The main analysis indicated no significant association between eNOS-4b/a polymorphism and the risk of DR in overall population [allelic model: OR = 0.94 (0.79–1.11); additive model: OR = 0.91 (0.73–1.14); recessive model: OR = 1.01 (0.81–1.25); dominant model: OR = 0.91 (0.75–1.09)]. Subgroup analysis by ethnicity also indicated no significant association. In conclusion, the current meta-analysis did not observe any association between the polymorphism of eNOS 4b/a and the risk of DR among type 2 diabetic subjects. However, larger well-designed studies are required to confirm this finding.

1. Introduction

Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes mellitus (DM) and a leading cause of adult blindness worldwide [1, 2]. Although long duration of diabetes and poor control of glycemia have been considered as the major risk factors for the development of DR, accumulated evidences suggest a genetic influence on susceptibility to this complication [3]. A number of genes have been suggested as candidate genes of diabetic retinopathy, for example, methylenetetrahydrofolate reductase gene, endothelial nitric oxide synthase gene (eNOS), vascular endothelial growth factor gene, and so on [4–6].

The eNOS gene is located on chromosome 7q35-36 and includes 26 exons, spanning 21 kb. The polymorphism of eNOS 4b/a gene consists of the two alleles of eNOS 4a with 4 tandem 27-repeats and eNOS 4b with 5 repeats [7]. NO is produced through the oxidation of L-arginine by eNOS [8]. NO can regulate endothelial function and is an important factor in the maintenance of homeostasis. NO can contribute to vasodilatation, increase local blood flow, and decrease vascular resistance in ocular circulation. Studies on humans and animal models have suggested that eNOS plays an essential role in retinal vascular function and disequilibrium in its production can lead to the development of DR [9–11]. The presence of eNOS polymorphisms might contribute to a decreased eNOS activity and a reduced NO level and has been reported to be a potential factor in the pathogenesis and development of DR.

To date, many case-control studies have been carried out to investigate the relationship between eNOS-4b/a polymorphism and the risk of DR among type 2 diabetic subjects, but results of these studies were conflicting and inconclusive. Some studies observed that there was an association between eNOS-4b/a polymorphism and the risk of DR [4, 12], while some others suggested there was no significant association [13, 14]. To draw a more reliable conclusion, we performed a meta-analysis of all available studies dealing with

the relationship between the eNOS-4b/a polymorphism and DR among type 2 diabetic subjects, including subgroup analyses based on different ethnicities.

2. Materials and Methods

2.1. Literature Search Strategy. We searched the literature databases including Pubmed, Embase, China National Knowledge Infrastructure (CNKI), and Chinese Wanfang Database. The last updated search was performed on November 20, 2013. The search used the following terms: "endothelial nitric oxide synthase or eNOS or 4b/a" in combination with "mutation or polymorphism or variant" and in combination with "diabetic retinopathy or DR." We also manually searched all the references of included studies to further identify additional relevant studies. Unpublished studies were not sought. For overlapping or republished studies, only the larger sample size or the most recent published papers were included in this meta-analysis. Publication language and publication date were not restricted in our search.

2.2. Inclusion and Exclusion Criteria. Studies included in this meta-analysis must meet the following criteria: (1) case-control or cohort studies; (2) studies evaluating the association between eNOS-4b/a polymorphism and DR risk; (3) subjects in the control group having type 2 diabetes but being free of DR; (4) human studies; and (5) having detailed data to calculate the odds ratio (OR) and 95% confidence interval (CI).

Studies were excluded if one of the following existed: (1) review articles or editorials; (2) case reports; (3) repeating or overlapping publications; (4) no report about the genotype frequency or insufficient information for data extraction.

2.3. Data Extraction. The following data were collected from each study: first author, publication date, region, ethnicity, sample size of cases and controls, genotype and allele frequencies of cases and controls, and genotyping methods. All the data were extracted independently by two investigators (Zejun Ma and Hui-Zhu Ren), according to the inclusion criteria above. Disagreements about eligibility were resolved through a discussion between the two investigators.

2.4. Statistical Analysis. In this meta-analysis, we evaluated the relationship between eNOS-4b/a polymorphism and DR risk using the allelic model (a versus b), the additive model (aa versus bb), the dominant model (aa + ab versus bb), and the recessive model (aa versus ab + bb) (see Figure 5). The alleles and genotypes between patients and control subjects were compared with OR and corresponding 95% CIs. A chi-square based Q statistics test and I^2 test were used to evaluate the heterogeneity between the studies ($P < 0.10$ and $I^2 > 50\%$ indicated the evidence of heterogeneity) [15]. The fixed effects model (FEM) was used when there was no statistical heterogeneity among the included studies; otherwise, the random effects model (REM) was used. Subgroup analysis was conducted according to different ethnicity. Sensitivity analysis was performed for estimating the stability of the meta-analysis. First, sensitivity analysis was carried out by exclusion of studies which failed the HWE test. Another analysis was done by omitting one study at a time to examine influence of one study on the overall summary estimate. Begg's funnel plot and Egger's test were carried out to assess possible publication bias [16, 17]. Asymmetric plot or the P value of Egger's test less than 0.05 suggested possible publication bias. Meta-analyses were performed using the statistical software Review Manager (version 5.2 for Windows, Cochrane Collaboration) and STATA software (version 12.0; Stata, College Station, TX), using two-sided P values.

3. Results

3.1. Characteristics of the Studies. Based on our search strategy, 61 potentially relevant articles were identified in Pubmed, Embase, CNKI, and Chinese Wanfang Database. A flow chart of study selection was shown in Figure 1. Of these, 46 were excluded because they did not meet the criteria or were overlapping publications. Finally, a total of 15 studies published between 2004 and 2012 met our inclusion criteria, involving 3,183 cases and 3,410 controls. The main characteristics of these studies were listed in Table 1. Of the ethnicity among all studies, four studies were performed on Caucasians [4, 13, 14, 18], ten were performed on Asian [12, 19–27], and one study was performed on West African [28]. The sample size in these studies varied considerably (ranging from 166 to 1446 individuals). The genotype and allele distributions for each study and HWE in controls were summarized in Table 2. Two genotyping methods were used to check genotypes in the studies including polymerase chain reaction (PCR) or PCR-restriction fragment length polymorphism (PCR-RFLP).

The distribution of the eNOS 4b/a genotype in control group was consistent with the HWE, except for two studies [23, 26]. Because excluding these two studies did not materially affect the results, they were still included in this analysis.

3.2. Meta-Analysis Results. The main results of meta-analysis for eNOS 4b/a polymorphism with the risk of DR were shown in Table 3. No significant association between eNOS 4b/a polymorphism and susceptibility to DR was identified in any of the genetic models (allelic model: OR = 0.94, 95% CI: 0.79–1.11; additive model: OR = 0.91, 95% CI: 0.73–1.14; the recessive model: OR = 1.01, 95% CI: 0.81–1.25; and the dominant model: OR = 0.91, 95% CI: 0.75–1.09) (Figures 2, 3, and 4).

In the subgroup analysis by ethnicity, similarly, there was still no significant association detected in all genetic models among Asians and Caucasians. The results of the subgroup analyses were shown in Table 3.

3.3. Heterogeneity and Publication Bias. Some heterogeneity was found during the course of the study. Hence, the random effects model was used. Begg's funnel plot and Egger's test were performed to assess the publication bias of the eligible literatures in this meta-analysis. The shapes of the funnel plots in all genetic models did not reveal any evidence of obvious asymmetry. The Egger's test further confirmed the absence

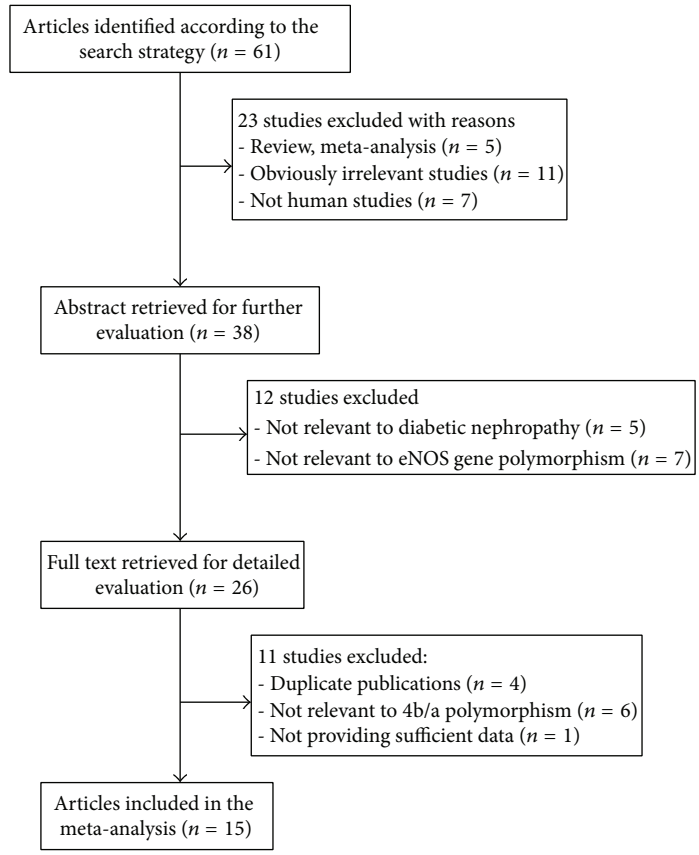

Figure 1: Flow chart of included studies.

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Odds ratio M-H, random, 95% CI
Awata et al. [12]	27	234	30	218	5.2%	0.82 [0.47, 1.43]
Cheema et al. [20]	518	1684	402	1208	11.0%	0.89 [0.76, 1.04]
Chen et al. [28]	37	136	232	602	7.1%	0.60 [0.39, 0.90]
Cilenšek et al. [4]	81	344	140	810	8.7%	1.47 [1.08, 2.01]
de Syllos et al. [14]	18	112	33	228	4.6%	1.13 [0.61, 2.11]
Ezzidi et al. [19]	149	766	246	978	9.9%	0.72 [0.57, 0.90]
Li et al. [26]	23	174	25	158	4.7%	0.81 [0.44, 1.49]
Mehrab-Mohseni et al. [21]	11	82	61	358	4.0%	0.75 [0.38, 1.51]
Petrovič et al. [18]	123	566	58	286	8.0%	1.09 [0.77, 1.55]
Santos et al. [13]	135	814	63	368	8.3%	0.96 [0.69, 1.34]
Suganthalakshmi et al. [22]	41	240	24	180	5.4%	1.34 [0.78, 2.31]
Sun et al. [24]	42	294	23	310	5.5%	2.08 [1.22, 3.55]
Uthra et al. [23]	68	374	59	376	7.5%	1.19 [0.81, 1.75]
Yue et al. [27]	31	308	53	334	6.2%	0.59 [0.37, 0.95]
Zhang et al. [25]	11	238	35	406	4.0%	0.51 [0.26, 1.03]
Total (95% CI)		**6366**		**6820**	**100.0%**	**0.94 [0.79, 1.11]**
Total events	1315		1484			

Heterogeneity: $\tau^2 = 0.06$; $\chi^2 = 38.38$, df = 14 ($P = 0.0005$); $I^2 = 64\%$
Test for overall effect: $Z = 0.70$ ($P = 0.48$)

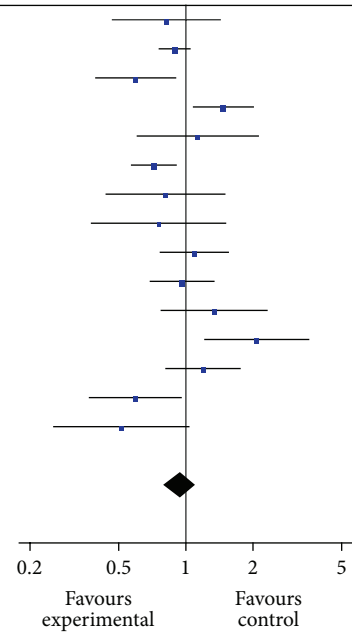

Figure 2: The forest plot of a versus b of eNOS polymorphism and overall DR risk.

Table 1: Main characteristics of included studies in the meta-analysis.

Author	Year	Country	Ethnicity	Case/control	Genotyping methods
Awata et al. [12]	2004	Japan	Asian	117/109	PCR
Cheema et al. [20]	2012	Indian	Asian	842/604	PCR-RFLP
Chen et al. [28]	2007	Ghana and Nigeria	West African	68/301	PCR
Cilenšek et al. [4]	2012	Slovenia	Caucasians	172/405	PCR
de Syllos et al. [14]	2006	Brazil	Caucasians	56/114	PCR
Ezzidi et al. [19]	2008	Bahrain	Asian	383/489	PCR-RFLP
Li et al. [26]	2010	china	Asian	87/79	PCR
Mehrab-Mohseni et al. [21]	2011	Iran	Asian	41/179	PCR
Petrovič et al. [18]	2008	Slovenia	Caucasians	283/143	PCR
Santos et al. [13]	2012	Brazil	Caucasians	407/184	PCR
Suganthalakshmi et al. [22]	2006	India	Asian	120/90	PCR-RFLP
Sun et al. [24]	2004	china	Asian	147/155	PCR-RFLP
Uthra et al. [23]	2007	India	Asian	187/188	PCR
Yue et al. [27]	1994	china	Asian	154/167	PCR
Zhang et al. [25]	2005	china	Asian	119/203	PCR

Table 2: The distribution of the 4b/a genotype and allele frequency for cases and controls.

Study	Distribution of 4b/a eNOS genotype						Allele frequency				HWE
	Cases			Controls			Cases		Controls		
	aa	ab	bb	aa	ab	bb	a	b	a	b	
Awata et al. [12]	1	25	91	3	24	82	27	207	30	188	Yes
Cheema et al. [20]	87	344	411	66	270	268	518	1166	402	806	Yes
Chen et al. [28]	10	17	41	48	136	117	37	99	232	370	Yes
Cilenšek et al. [4]	15	51	106	13	114	278	81	263	140	670	Yes
de Syllos et al. [14]	1	16	39	3	27	84	18	94	33	195	Yes
Ezzidi et al. [19]	17	115	251	33	180	276	149	617	246	732	Yes
Li et al. [26]	5	13	69	7	11	61	23	151	25	133	No
Mehrab-Mohseni et al. [21]	12	111	284	5	53	126	135	679	63	305	Yes
Petrovič et al. [18]	16	91	176	7	44	92	123	443	58	228	Yes
Santos et al. [13]	12	111	284	5	53	126	135	679	63	305	Yes
Suganthalakshmi et al. [22]	7	27	86	3	18	69	41	199	24	156	Yes
Sun et al. [24]	3	36	108	1	21	133	42	252	23	287	Yes
Uthra et al. [23]	7	54	126	1	57	130	68	306	59	317	No
Yue et al. [27]	2	27	125	4	45	118	31	277	53	281	Yes
Zhang et al. [25]	0	11	108	2	31	170	11	227	35	371	Yes

HWE: Hardy-Weinberg equilibrium, $P < 0.05$ was considered significant.

of publication bias for any of the four genetic models in this meta-analysis ($P = 0.277$ for dominant model, $P = 0.313$ for recessive model, $P = 0.287$ for additive model, and $P = 0.764$ for allelic model, resp.).

3.4. Sensitivity Analysis. Sensitivity analyses were performed to assess the stability of the results. Although two studies [23, 26] did not follow the HWE, the summary ORs were not materially altered including or excluding the studies (data shown in Table 3). Moreover, no other single study influenced the overall results (data not shown), which indicated that our results were statistically reliable and robust.

4. Discussion

The polymorphism of eNOS-4b/a gene has been associated with many vascular diseases including hypertension, diabetic retinopathy, and diabetic nephropathy in various populations [29, 30]. Variable results have been reported for the association of eNOS-4b/a polymorphism with DR [13, 20, 21]. In a systematic meta-analysis study [3], no relationship of the eNOS 4b/a polymorphism was found with DR development regardless of ethnicity. However, a recent meta-analysis study found that eNOS 4b/a has a protective effect against DR [31]. The present meta-analysis of 15 studies, including 3,183 cases and 3,410 controls, provided the most comprehensive analysis on the association of the eNOS 4b/a polymorphism

Table 3: Meta analysis of the association of eNOS-4b/a gene polymorphism with DR in type 2 diabetes.

Genetic model	Populations	Studies (n)	Number of cases/controls	Heterogeneity Q test P-value	I^2 (%)	OR (95% CI)	P value
a versus b	All	15	3183/3410	0.0005	64	0.94 (0.79–1.11) (REM)	0.48
	Asian	10	2265/2564	0.006	61	0.90 (0.73–1.11) (REM)	0.32
	Caucasians	4	918/846	0.30	18	1.17 (0.97–1.40) (FEM)	0.10
	HWE (yes)	13	2909/3143	0.0003	67	0.93 (0.77–1.12) (REM)	0.44
aa versus bb	All	15	3183/3410	0.04	43	0.91 (0.73–1.14) (FEM)	0.40
	Asian	10	2265/2564	0.21	24	0.78 (0.61–1.01) (FEM)	0.06
	Caucasians	4	918/846	0.26	25	1.64 (0.98–2.73) (FEM)	0.06
	HWE (yes)	13	2909/3143	0.06	41	0.88 (0.70–1.11) (FEM)	0.28
aa + ab versus bb	All	15	3183/3410	0.002	60	0.91 (0.75–1.09) (REM)	0.30
	Asian	10	2265/2564	0.0004	61	0.82 (0.65–1.04) (REM)	0.11
	Caucasians	4	918/846	0.58	0	1.13 (0.91–1.40) (FEM)	0.26
	HWE (yes)	13	2909/3143	0.0007	65	0.89 (0.72–1.10) (REM)	0.30
aa versus bb + ab	All	15	3183/3410	0.21	22	1.01 (0.81–1.25) (FEM)	0.96
	Asian	10	2265/2564	0.49	0	0.90 (0.70–1.15) (FEM)	0.39
	Caucasians	4	918/846	0.28	21	1.60 (0.97–2.65) (FEM)	0.07
	HWE (yes)	13	2909/3143	0.30	14	0.98 (0.78–1.23) (FEM)	0.88

OR: odds ratio; CI: confidence interval.

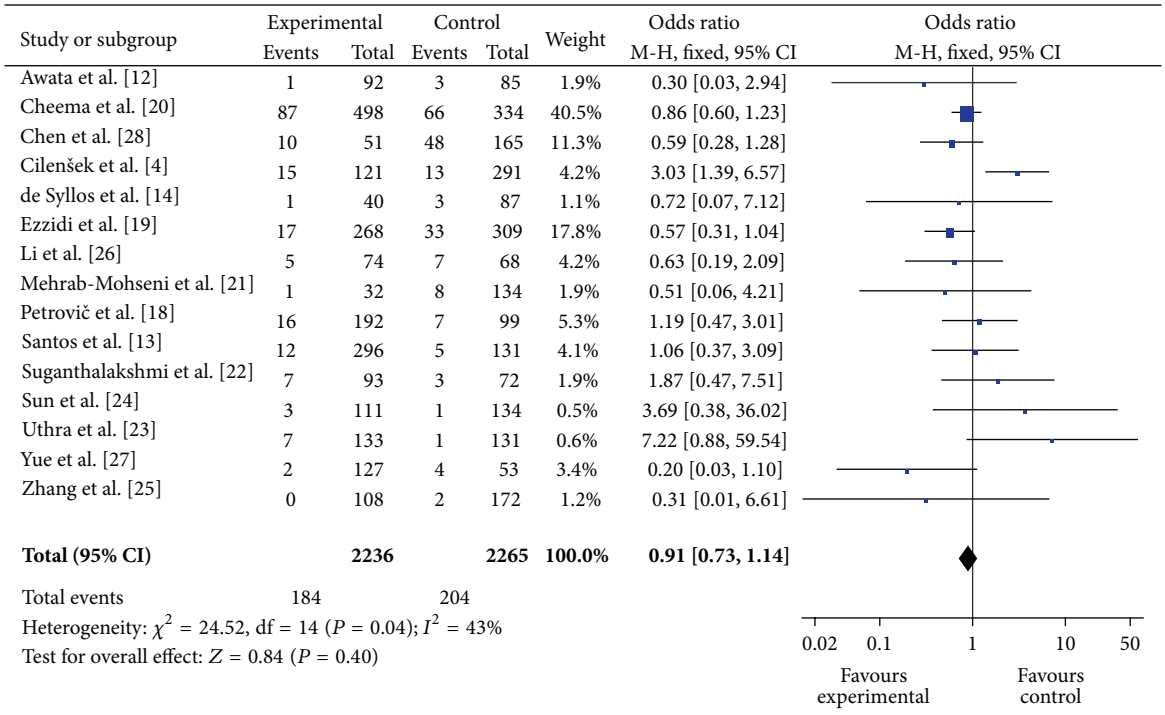

Figure 3: The forest plot of aa versus bb of eNOS polymorphism and overall DR risk.

with the risk of diabetic retinopathy. The results indicated that the eNOS 4b/a polymorphism was not associated with an increased risk of DR in the overall studied population. These findings were consistent with most of the studies that were included in our meta-analysis [13, 14, 23]. The lack of association between eNOS 4b/a polymorphism and diabetic retinopathy suggested that genetic variations in the eNOS 4b/a gene did not predict the risk of diabetic retinopathy in T2DM patients. It is possible that eNOS-derived NO plays a minor role in the development of diabetic retinopathy. In the subgroup analysis according to ethnicity, no significant association was observed in Asian and Caucasians in all genetic models. The results demonstrated that ethnic difference in genetic background and the living environment

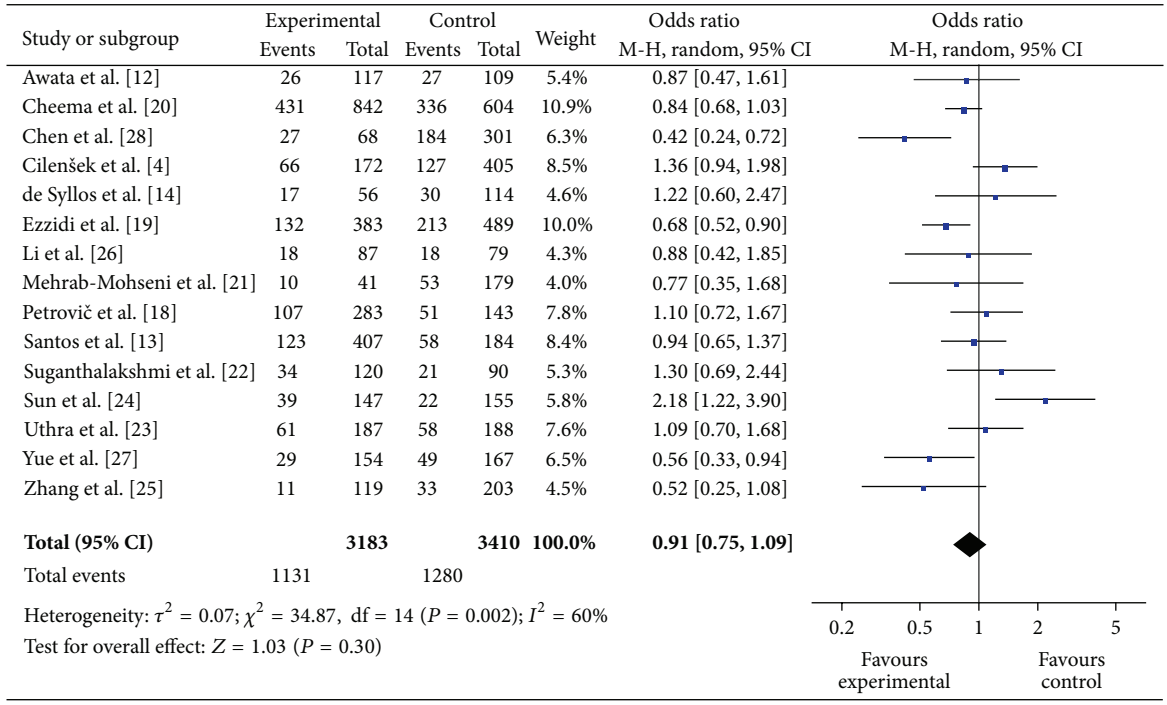

Figure 4: The forest plot of aa + ab versus bb of eNOS polymorphism and overall DR risk.

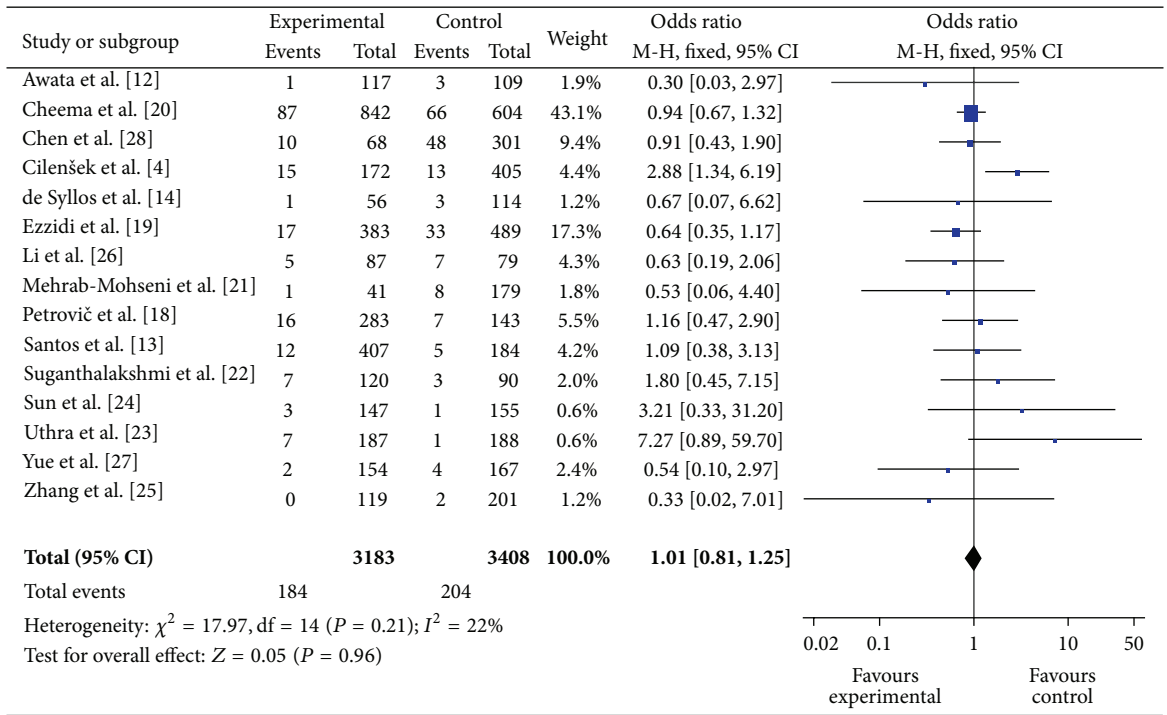

Figure 5: The forest plot of aa versus bb + ab of eNOS polymorphism and overall DR risk.

did not play an obvious role in the association between the eNOS 4b/a polymorphism and the risk of DR. Although the available genetic data do not implicate the eNOS 4b/a polymorphism as a determinant of DR susceptibility in Asian and Caucasians, further studies are needed to see if the eNOS 4b/a polymorphism can confer a risk of DR in other ethnic populations.

Publication bias is an important factor affecting us to get a reliable conclusion for meta-analysis. In this meta-analysis, no significant publication bias for 4b/a polymorphism in

any of the above-mentioned inherited models was found, suggesting that the results observed should be stable. Sensitivity analyses did not significantly alter the results, also suggesting that our results were statistically reliable and stable.

Our study has several limitations that need to be taken into consideration when interpreting the results. First, significant between-study heterogeneity was detected in some comparisons and might distort the meta-analysis. Second, only published studies in English or Chinese were included for data analysis; some potential studies with other languages or unpublished could be missed. Third, this meta-analysis was based predominantly on Asian research. Only 4 studies involving Caucasians and one study involving Africans were included. No study from other parts of the world was found. This may develop a partial result. Fourth, our meta-analysis was based on unadjusted OR estimates. Despite these limitations, our study provides a better understanding of the association between eNOS-4b/a gene polymorphisms and risk of DR in type 2 diabetes.

In summary, this meta-analysis indicates that the eNOS 4b/a polymorphism is not associated with an increased risk of DR among type 2 diabetic subjects. Taking into account the limitations of this meta-analysis, further larger well-designed studies involving different ethnic populations, particularly referring to gene-gene and gene-environment interactions, are required to confirm this finding.

Conflict of Interests

All authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Ze-jun Ma and Li-ming Chen conceived and designed the experiments. Ze-jun Ma, Xin Guo, and Rui Chen carried out the literature searching and data extraction. Ze-jun Ma, Hui-Zhu Ren, Jun Guo, and Li-ming Chen analyzed the data. Ze-jun Ma, Xin Guo, Rui Chen, and Jun Guo contributed reagents/materials/analysis tools. Ze-jun Ma, Rui Chen, and Li-ming Chen wrote the paper.

Acknowledgment

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Review Article

The Associations between VEGF Gene Polymorphisms and Diabetic Retinopathy Susceptibility: A Meta-Analysis of 11 Case-Control Studies

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Aims. Published data on the associations of VEGF polymorphisms with diabetic retinopathy (DR) susceptibility are inconclusive. A systematic meta-analysis was undertaken to clarify this topic. *Methods*. Data were collected from the following electronic databases: PubMed, Embase, OVID, Web of Science, Elsevier Science Direct, Excerpta Medica Database (EMBASE), and Cochrane Library with the last report up to January 10, 2014. ORs and 95% CIs were calculated for VEGF–2578C/A (rs699947), –1154G/A (rs1570360), –460T/C (rs833061), −634G>C (rs2010963), and +936C/T (rs3025039) in at least two published studies. Meta-analysis was performed in a fixed/random effect model by using the software STATA 12.0. *Results*. A total of 11 studies fulfilling the inclusion criteria were included in this meta-analysis. A significant relationship between VEGF+936C/T (rs3025039) polymorphism and DR was found in a recessive model (OR = 3.19, 95% CI = 1.20–8.41, and $P(z) = 0.01$) in Asian and overall populations, while a significant association was also found between –460T/C (rs833061) polymorphism and DR risk under a recessive model (OR = 2.12, 95% CI = 1.12–4.01, and $P(z) = 0.02$). *Conclusions*. Our meta-analysis demonstrates that +936C/T (rs3025039) is likely to be associated with susceptibility to DR in Asian populations, and the recessive model of –460T/C (rs833061) is associated with elevated DR susceptibility.

1. Introduction

Type 2 diabetes (T2DM) is a metabolic disorder that has caused major public health threat throughout the world. Diabetic retinopathy (DR), one of the most prominent pathological microvascular complications of T2DM, is also the leading cause of legal blindness in working-age adults [1], but its frequency varies in different ethnicities.

Hyperglycemia has been regarded as the dominant pathogenic factor in the development and progression of DR [2]. The Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) showed that 28.8% of diabetic patients develop retinopathy early, whereas 22.2% with the history of diabetes irrespective of glycemic exposure do not develop retinopathy [3]. This study suggested that genetic factors could facilitate the happening of retinopathy in diabetic patients.

Currently, the pathogenesis of DR is considered to be influenced by environmental and genetic factors. Ethnic differences in the prevalence of DR may offer understandings into the relative importance of genetic or environmental risk factors. Therefore, it is important to identify molecular markers that may help in the diagnosis of DR in multiple populations.

Diabetic retinopathy is characterized by vascular permeability, increased tissue ischemia, and angiogenesis. Vascular endothelial growth factor (VEGF), a potent angiogenic and vascular permeability factor [4], plays a significant role

through inducing hyperpermeability of retinal vessels, breakdown of the blood-retinal barrier, and neovascularization [5–7]; moreover, VEGF antagonists are able to reduce retinal vascular permeability and neovascularization, thus inhibiting the development of DR [8, 9]; therefore VEGF may be strongly implicated in the progression of DR.

The human VEGF gene is located on chromosome 6 (6p21.3) and highly polymorphic, especially in the promoter, the 5′-untranslated (UTR) and the 3′-untranslated region. The promoter has a single transcription start site near to a group of Sp1 binding sites and covers AP-1 and AP-2 binding sites [10], while the 3′-UTR of VEGF gene is predicted to embrace mRNA destabilizing elements which reduces VEGF mRNA under normoxic conditions and revealed to be acting in conjunction with the 5′-UTR and coding region of the gene to bring about mRNA stability during hypoxia [11].

Until now, the exact pathogenesis of DR is not yet fully clarified, despite a large number of studies on the candidate genes for the DR susceptibility in subjects of various ethnicities; however, most of the data appear to be inconclusive and require further confirmation. This may be attributed to the limited sample size and inadequate statistical power and studies with a relatively small sample size, which may have affected their reliability. Meta-analysis provides the most accurate estimate of the nature and magnitude of an effect by combining the results of multiple independent studies and has the ability to reduce the potential influence of types I and II errors that appear within individual studies [12]. Therefore, we performed a comprehensive meta-analysis to evaluate and confirm the associations of VEGF gene polymorphisms with DR susceptibility; we focused on the promoter region (−2578C/A rs699947, −1154G/A rs1570360), 5′-UTR (−634G>C rs2010963, −460T/C rs833061), and the 3′-UTR(+936C/T rs3025039) as it has been shown to be highly polymorphic and the most studied polymorphisms.

2. Subjects and Methods

2.1. Identification and Eligibility of Relevant Studies of Meta-Analysis. A systematic literature search in PubMed, Embase, OVID, Web of Science, Elsevier Science Direct, Excerpta Medica Database (EMBASE), and Cochrane Library (last search updated on January 10, 2014) was carried out to identify studies involving the associations between DR and the above mentioned VEGF polymorphisms. The language was limited to English. In this meta-analysis, the controls were patients with T2DM without DR (DWR) and the cases were T2DM patients with DR (all retinopathy, including NPDR-nonproliferative diabetic retinopathy and PDR-proliferative diabetic retinopathy). We conducted subgroup analyses stratified by status of DR (based on the studies that had defined cases solely in the presence of PDR or NPDR) [13].

The search terms were as follows: "vascular endothelial growth factor or VEGF" in integration with "polymorphism or mutation or variant" and in integration with "diabetic retinopathy or DR or NPDR or PDR" to identify all publications, which investigated the associations of the VEGF polymorphisms with DR risk in all ethnic populations. Meanwhile, we also searched the reference lists of included studies to identify other potentially eligible studies. Case reports, editorials, and reviews were excluded.

Studies included in our meta-analysis must meet the following criteria: (1) the article pertained to the above mentioned VEGF polymorphisms and DR risk; (2) sufficient data for examining odds ratios (ORs) with 95% confidence intervals (CIs); (3) genotype distributions of polymorphism of the control population were consistent with Hardy-Weinberg equilibrium (HWE).

Two investigators independently extracted data. From each study, we extracted the first author's name, year of publication, country of origin, ethnicity of samples, number of cases and controls, and the available genotype frequency of the polymorphisms. Ethnicity was classified as White or Asian. The control group sources were classified as population-based or hospital-based controls. The deviation of the genotype frequencies in the control population from HWE was calculated separately for each study.

2.2. Statistical Analysis. We conducted the meta-analysis using STATA software (version 12; Stata Corporation, College Station, Texas). ORs and 95% CIs were calculated to assess the strength of the associations between the VEGF polymorphisms and DR susceptibility. The pooled OR was calculated for the codominant model, dominant model, recessive model, and additive model, respectively. $P < 0.05$ was considered statistically significant.

The inconsistency index, I^2, was calculated to identify the heterogeneity [14]. The data were used with the fixed effects pooling model if there was no heterogeneity ($I^2 < 50\%$). Alternatively, the random effects model was used ($I^2 > 50\%$). If there is heterogeneity, the Galbraith graph was used to explore the potential sources of heterogeneity. We assessed the potential publication bias with funnel plots of the effect sizes versus the standard errors and identified the significant asymmetry by the Begg's test [15]. To test for funnel plot asymmetry, Egger's test was also performed [15]. The leave-one-out sensitivity was performed, in which the meta-analysis estimates were computed after every study being omitted in each turn [16].

3. Results

3.1. Characteristics of All Included Studies. The initial search yielded 259 references. Based on titles and/or abstracts, we excluded 202 and reviewed 57 full-text reports. Applying the study inclusion criteria, 11 studies were included in this meta-analysis (Figure 1). The study selection procedure was showed in Figure 1, and the study characteristics were displayed in Tables 1 and 2.

Seven relevant studies with a total number of 1,085 cases and 1,019 controls were included in −634G>C (rs2010963) analysis [17–23]; 6 relevant studies with a total number of 887 cases and 981 controls were included in −2578C/A (rs699947) analysis [17, 20–24]; 4 relevant studies with a total number of 531 cases and 616 controls were included in +936C/T

Table 1: Characteristics of all studies assessing the relationship between VEGF and diabetic retinopathy (DR versus DWR).

First author	Year	Country/racial decent	Study design	Cases[a] (DR)	Controls[a] (DWR)	Cases			Controls			HWE
						11[b]	12[b]	22[b]	11[b]	12[b]	22[b]	X^2 control population
−634 G>C (rs2010963)												
Awata [17]	2005	Japan (Asian)	Hospital based controls	175	203	46	91	38	75	95	30	0.99
Globočnik Petrovič [18]	2008	Slovene (Caucasian)	Hospital based controls	206	143	79	103	24	61	67	15	0.58
Uthra [19]	2008	India (Asian)	Population based controls	131	82	60	51	9	44	29	6	0.69
Nakamura [20]	2009	Japan (Asian)	Hospital based controls	177	292	63	79	34	84	146	59	0.75
Chun [21]	2010	Korea (Asian)	Hospital based controls	253	134	85	125	43	43	69	22	0.51
Yang [22]	2011	China (Asian)	Hospital based controls	129	139	36	74	19	39	72	26	0.47
Bleda [23]	2012	Spain (Caucasian)	Hospital based controls	14	26	7	5	2	10	11	5	0.53
−2578 C/A (rs699947)												
Awata [17]	2005	Japan (Asian)	Hospital based controls	175	203	95	70	10	93	91	19	0.62
Abhary [24]	2009	Australia (Caucasian)	Hospital based controls	139	187	31	74	31	45	91	45	0.94
Nakamura [20]	2009	Japan (Asian)	Hospital based controls	177	292	85	70	22	163	107	22	0.44
Chun [21]	2010	Korea (Asian)	Hospital based controls	253	134	123	115	15	92	36	6	0.31
Yang [22]	2011	China (Asian)	Hospital based controls	129	139	66	47	16	82	51	5	0.38
Bleda [23]	2012	Spain (Caucasian)	Hospital based controls	14	26	0	12	2	8	10	8	0.23
+936 C/T (rs3025039)												
Awata [25]	2002	Japan (Asian)	Hospital based controls	150	118	93	47	10	85	31	2	0.66
Uthra [19]	2008	India (Asian)	Population based controls	131	82	111	19	0	65	17	0	0.29
Kim [26]	2009	Korea (Asian)	Population based controls	121	277	55	63	3	226	51	0	0.09
Yang [22]	2011	China (Asian)	Hospital based controls	129	139	83	42	3	89	47	3	0.25
−460 T/C (rs833061)												
Awata [25]	2002	Japan (Asian)	Hospital based controls	150	118	79	58	13	52	57	9	0.21
Suganthalakshmi [27]	2006	India (Asian)	Hospital based controls	120	90	36	81	3	61	29	0	0.06
Yang [22]	2011	China (Asian)	Hospital based controls	129	139	65	46	16	81	52	5	0.33

DR: diabetic retinopathy; DWR: diabetic without retinopathy; HWE: Hardy-Weinberg equilibrium.

[a]Sample size.

[b]11: wild-type homozygote; 12: heterozygote; 22: variant homozygote.

Table 2: Characteristics of all studies assessing the relationship between VEGF and diabetic retinopathy (PDR versus NPDR).

First author	Year	Country/racial decent	Study design	Cases[a] (PDR)	Controls[a] (NPDR)	Cases			Controls		
						11[b]	12[b]	22[b]	11[b]	12[b]	22[b]
−634 G>C (rs2010963)											
Awata [25]	2002	Japan (Asian)	Hospital based controls	70	80	24	30	16	14	51	15
Uthra [19]	2008	India (Asian)	Population based controls	44	87	21	17	3	39	33	7
Chun [21]	2010	Korea (Asian)	Hospital based controls	145	108	48	73	24	37	52	19
+936 C/T (rs3025039)											
Awata [25]	2002	Japan (Asian)	Hospital based controls	70	80	44	20	6	49	27	4
Uthra [19]	2008	India (Asian)	Population based controls	44	87	35	9	0	76	10	0
Kim [26]	2009	Korea (Asian)	Population based controls	37	84	15	19	3	40	44	15

PDR: proliferative diabetic retinopathy; NPDR: nonproliferative diabetic retinopathy.

[a]Sample size.

[b]11: wild-type homozygote; 12: heterozygote; 22: variant homozygote.

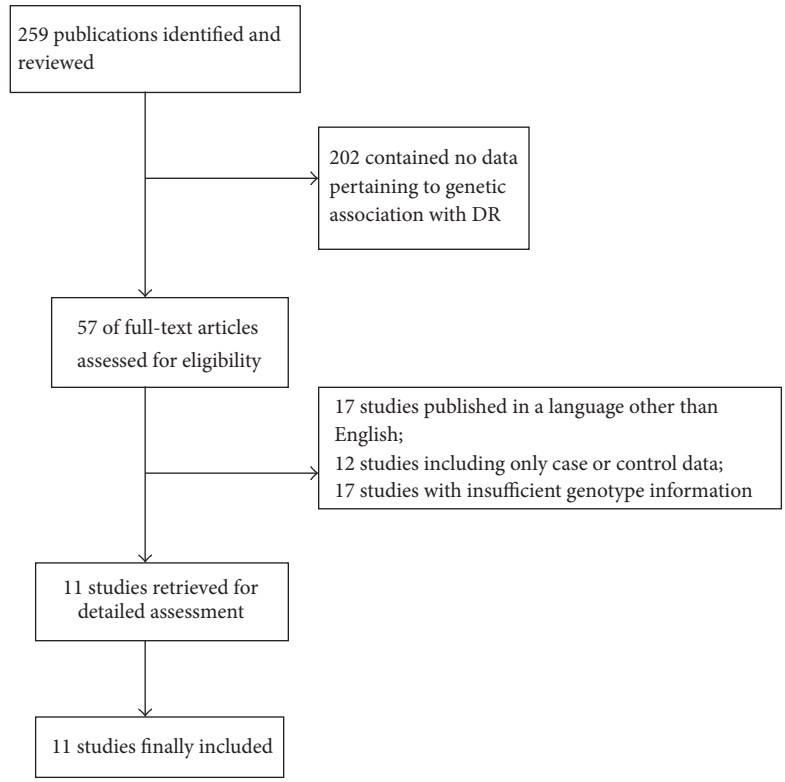

Figure 1: Flow chart of study selection process and included studies.

(rs3025039) analysis [19, 22, 25, 26], while 3 relevant studies with a total number of 399 cases and 347 controls were included in −460T/C (rs833061) analysis [22, 25, 27] (Table 1). The distributions of the genotypes in the control populations were consistent with HWE in all of the studies.

Of the 11 studies, 9 were hospital-based control studies [17, 18, 20–25, 27], and 2 were population-based control studies [19, 26]. A total of 8 studies included Asian individuals [17, 19, 26] and 3 included White individuals [18, 23, 24].

Three relevant studies were included in −634G>C (rs2010963) analysis (PDR versus NPDR), and 3 relevant studies were included in +936C/T (rs3025039) analysis (PDR versus NPDR) (Table 2).

3.2. Pooled Effects for the VEGF −634G>C (rs2010963) Polymorphism and DR Risk (DR versus DWR). Analyses were performed for all cases with any form of DR compared with all diabetics without retinopathy (DWR). The summary results of meta-analysis for VEGF gene polymorphisms and DR risk were shown in Table 3. No significant association was detected under all genetic models in the overall populations and subgroup analysis for −634G/C (rs2010963) polymorphism (Table 3).

3.3. Pooled Effects for the VEGF −2578C/A (rs699947) Polymorphism and DR Risk (DR versus DWR). Our meta-analysis did not show any significant correlations between −2578C/A (rs699947) and DR risk in hospital-based control studies, overall populations, and Asians populations, respectively (Table 3).

3.4. Pooled Effects for the VEGF +936C/T (rs3025039) Polymorphism and DR Risk (DR versus DWR). Our data demonstrated that +936C/T (rs3025039) polymorphism increased the DR risk in the Asian populations (recessive model, OR = 3.19, 95% CI = 1.20–8.41, and $P(z) = 0.01$). The effects of the ORs and 95 CIs of the Asian populations and overall populations were the same, because all of the studies included in the +936C/T (rs3025039) were all Asian populations (Table 3).

3.5. Pooled Effects for the VEGF −460T/C (rs833061) Polymorphism and DR Risk (DR versus DWR). A significant association between the −460T/C (rs833061) polymorphism and increased DR risk was detected under a recessive model (OR = 2.12, 95% CI = 1.12–4.01, and $P(z) = 0.02$) (Table 3).

3.6. Pooled Effects for the VEGF −1154G/A (rs1570360) Polymorphism and DR Risk (DR versus DWR). There were only two studies included in the −1154G/A (rs1570360) meta-analysis; therefore the −1154G/A (rs1570360) was not further analyzed.

3.7. Pooled Effects for the VEGF −634G>C (rs2010963), +936C/T (rs3025039) Polymorphism and DR Risk (PDR versus NPDR). There were only three studies included

Table 3: Meta-analysis of the associations between the VEGF polymorphisms and DR risk (DR versus DWR).

Variables	n^a	Variant homozygote versus wild-type homozygote				Heterozygote versus wild-type homozygote				Dominant model				Recessive model				Additive model			
		OR (95% CI)	$P(z)$	I^2 (%)	Egger's test P	OR (95% CI)	$P(z)$	I^2 (%)	Egger's test P	OR (95% CI)	$P(z)$	I^2 (%)	Egger's test P	OR (95% CI)	$P(z)$	I^2 (%)	Egger's test P	OR (95% CI)	$P(z)$	I^2 (%)	Egger's test P
−634 G>C (rs2010963)																					
Overall	7	1.07 (0.81–1.40)	0.61	17.8	0.72	1.06 (0.87–1.29)	0.54	17.6	0.88	1.04 (0.86–1.24)	0.67	30.9	0.85	1.05 (0.82–1.33)	0.69	0	0.55	1.04 (0.92–1.19)	0.47	28.5	0.80
Asian	5	1.06 (0.71–1.58)	0.75	40.6	0.97	1.04 (0.84–1.30)	0.68	39.2	0.40	1.07 (0.79–1.44)	0.66	50.6	0.53	1.04 (0.80–1.36)	0.71	0	0.75	1.04 (0.85–1.27)	0.66	48.3	0.78
Hospital based	6	1.07 (0.81–1.41)	0.63	31.5	0.71	1.03 (0.84–1.27)	0.72	26.7	0.79	1.04 (0.86–1.27)	0.63	42.2	0.74	1.05 (0.82–1.35)	0.67	0	0.58	1.03 (0.91–1.18)	0.57	39.1	0.71
−2578 C/A (rs699947)																					
Overall	6	1.47 (0.81–2.67)	0.20	59.6	0.47	1.31 (0.87–1.96)	0.19	70.0	0.34	1.33 (0.89–1.99)	0.15	72.1	0.44	1.17 (0.68–1.99)	0.56	58.9	0.98	1.24 (0.92–1.66)	0.14	72.2	0.62
Asian	4	1.59 (0.71–3.56)	0.25	71.7	0.77	1.26 (0.79–2.01)	0.32	77.3	0.73	1.32 (0.82–2.13))	0.24	80.3	0.69	1.45 (0.71–2.95)	0.29	65.1	0.80	1.29 (0.87–1.92)	0.19	81.5	0.54
Hospital based	6	1.47 (0.81–2.67)	0.20	59.6	0.47	1.31 (0.87–1.96)	0.19	70	0.34	1.33 (0.89–1.99)	0.15	72.1	0.44	1.17 (0.68–1.99)	0.56	58.9	0.98	1.24 (0.92–1.66)	0.14	72.2	0.62
+936 C/T (rs3025039)																					
Overall	4	3.73 (0.76–18.25)	0.10	50.7	0.48	1.47 (0.59–3.66)	0.40	90.8	0.29	1.54 (0.62–3.85)	0.34	91.2	0.33	3.19 (1.20–8.41)	0.01*	32.7	0.53	1.49 (0.71–3.12)	0.28	89.7	0.34
Asian	4	3.73 (0.76–18.25)	0.10	50.7	0.48	1.47 (0.59–3.66)	0.40	90.8	0.29	1.54 (0.62–3.85)	0.34	91.2	0.33	3.19 (1.20–8.41)	0.01*	32.7	0.53	1.49 (0.71–3.12)	0.28	89.7	0.34
−460 T/C (rs833061)																					
Overall	3	2.46 (0.66–9.11)	0.17	64	0.56	1.50 (0.50–4.51)	0.46	92.1	0.11	1.57 (0.51–4.89)	0.42	93	0.27	2.12 (1.12–4.01)	0.02*	42.4	0.66	1.53 (0.76–3.07)	0.22	88.3	0.25

[a]Number of studies.

$P(z)$: z test used to determine the significance of the overall OR.

I^2: inconsistency index; random-effects model was used when I^2 test >50%; otherwise, fix-effects model was used.

NA: Not available.

*Significant results.

in −634G>C (rs2010963) and +936C/T (rs3025039) meta-analysis (PDR versus NPDR) (Table 2). Our meta-analysis did not show any significant correlations between VEGF −634G>C (rs2010963), +936C/T (rs3025039), and DR risk, respectively (PDR versus NPDR) (Table 4).

3.8. Heterogeneity Analysis. In Table 3, we have noticed that most of the comparisons had significant heterogeneity, we therefore conducted subgroup analysis with available studies, the Asian populations and hospital-based studies were mainly the sources of heterogeneity. However, in the significant comparisons, recessive model in Asian populations of the +936C/T (rs3025039) polymorphism, and recessive model of −460T/C (rs833061), heterogeneity was not found (Table 3).

3.9. Publication Bias. Publication bias was examined by funnel plots qualitatively and assessed by Egger's tests quantitatively. The results of Egger's regression test showed that there was no publication bias for the significant comparisons ($P = 0.53$ for the recessive model of +936C/T (rs3025039) in Asian populations; $P = 0.66$ for the recessive model of −460T/C (rs833061)) (Table 3).

Begg's test and Egger's test did not detect any significantly statistical evidence of publication bias for any of the genetic models. This indicates that the results of this meta-analysis are relatively stable and that publication bias is unlikely to have affected the results.

3.10. Sensitivity Analysis. We performed the sensitivity analyses by sequentially removing individual eligible study. The results indicated that the overall significance of the ORs was not altered by any single study for the recessive model of the +936C/T (rs3025039) polymorphism in Asian populations and −460T/C (rs833061) (data not shown). The sensitivity analyses also indicate that results of our study are stable and reliable.

4. Discussion

To our best of knowledge, this is the first meta-analysis involving the five VEGF polymorphisms at the same time. Our meta-analysis finds significant associations between +936C/T (rs3025039) and DR susceptibility in Asian populations, while Kim et al. found that +936C/T (rs3025039) polymorphism was related with DR in Korean populations [26]; however Awata et al. demonstrated nonsignificant association of this polymorphism with the progression of DR in Japanese [25]. The main reason for this discrepancy might be racial differences in the studied populations. Other reasons might be due to differences in the inclusion criteria of cases, sampling bias, sample sizes, and so forth.

The most investigated VEGF polymorphism has been −634G>C (rs2010963), with most studies showing no significant association between this polymorphism and the presence of DR; as well, the present meta-analysis confirms the nonsignificant association across all of the overall and subgroup analysis, which is consistent with the results of T. Zhao and J. Zhao meta-analysis [28]. Although we reached the same conclusion, there are some differences between us. First, eight studies were included in T. Zhao and J. Zhao meta-analysis, three of them deviated from HWE [27, 29, 30], therefore resulting in obvious heterogeneity. Second, an expanding body of literature on this topic has been published since 2010, but unfortunately was not included in the T. Zhao and J. Zhao meta-analysis. Besides, T. Zhao and J. Zhao also included the study conducted by Kangas-Kontio et al. [31], which was inappropriate, because the diabetic patients also included type 1 diabetes in that study. We believe our results are more reliable and stable based on the sample size, thoughtful design, and strict criterion for the included studies.

Contrary to our meta-analysis, Qiu et al. conducted a similar meta-analysis only on −634G>C polymorphism and DR, which involved a total of 1525 DR cases and 1422 DWR controls in 9 independent studies, they observed a significant relationship between −634G>C (rs2010963) polymorphism and DR in an allelic genetic model (OR: 1.13) and a recessive genetic model (OR: 1.26). However, the genotypes deviation of the controls in Yang et al.'s study was not consistent with HWE [32] but was still included in Qiu et al. meta-analysis [33], though they stated that all the studies included were not deviated from HWE. Moreover, the study performed by Errera et al. was inappropriate to include in their meta-analysis, because the T2DM patients were divided into patients with PDR and patients without PDR in that study [34]; as we all know, DR consists of PDR and NPDR, the cases and controls were DR and DWR in Qiu et al. meta-analysis respectively, and a significant association was evidenced in Errera et al. study (with the second largest weight from the forest plot) in Qiu et al. meta-analysis; therefore the results of Qiu et al. meta-analysis maybe biased and unreliable.

Some possible limitations of our meta-analysis should be taken into consideration. First, the conclusion was based on a relatively small number of participants. Second, potential publication biases may exist in this meta-analysis because studies excluded the non-English-language publications. Third, this meta-analysis was based on unadjusted data due to a lack of detailed genotype information stratified by many variables (gender, age, etc.) in original articles, and a more precise analysis would have been performed if all individual raw data had been available.

In spite of these potential limitations, our meta-analysis has some strength. First, we sought to find as many publications as we could by searching various databases. The sufficient number of cases and controls were pooled from multiple studies, which apparently increased the statistical power of our analysis. Second, in order to minimize the potential bias, we designed a rigorous protocol and utilized explicit methods for the literature search, study selection, data extraction, and statistical analysis. The symmetry of the funnel plot suggests that bias is less likely to have appeared, indicating that the pooled results of our analysis may be unbiased. Third, no publication bias was detected among the pooled results. Last, we considered not only association between the most investigated −634G>C (rs2010963) and DR

Table 4: Meta-analysis of the associations between the VEGF polymorphisms and DR risk (PDR versus NPDR).

Variables	n[a]	Variant homozygote versus wild-type homozygote				Heterozygote versus wild-type homozygote				Dominant model				Recessive model				Additive model			
		OR (95% CI)	$P(z)$	I^2 (%)	Egger's test P	OR (95% CI)	$P(z)$	I^2 (%)	Egger's test P	OR (95% CI)	$P(z)$	I^2 (%)	Egger's test P	OR (95% CI)	$P(z)$	I^2 (%)	Egger's test P	OR (95% CI)	$P(z)$	I^2 (%)	Egger's test P
−634 G>C (rs2010963)																					
Overall	3	0.62 (0.36–1.06)	0.08	0	0.63	0.73 (0.36–1.46)	0.37	64.5	0.52	0.76 (0.43–1.34)	0.35	52.9	0.51	1.02 (0.63–1.65)	0.90	0	0.84	0.91 (0.70–1.17)	0.47	0	0.70
+936 C/T (rs3025039)																					
Overall	3	0.93 (0.37– 2.33)	0.89	27.1	NA	1.11 (0.70– 1.77)	0.64	0	0.05	1.1 (0.70– 1.72)	0.66	0	0.19	0.89 (0.37–2.13)	0.80	46.3	NA	1.04 (0.73–1.5)	0.80	0	0.29

[a]Number of studies.

$P(z)$: z test used to determine the significance of the overall OR.

I^2: inconsistency index; random-effects model was used when I^2 test <50%; otherwise, fix-effects model was used.

NA: not available.

susceptibility but also paid attention to the impact of other VEGF polymorphisms −2578C/A (rs699947), −1154G/A (rs1570360), −460T/C (rs833061), and +936C/T (rs3025039). We could therefore give a more complete picture on the role of VEGF polymorphisms contributing to DR risk.

In conclusion, our meta-analysis revealed some significant associations between VEGF polymorphisms and DR susceptibility. However due to the relatively small sample size in this meta-analysis, in order to reach a more definitive conclusion, further studies based on larger sample size and substantiation of the variations through functional studies are still needed.

Conflict of Interests

The authors declare that there is no conflict of interests or financial interests associated with this paper.

Authors' Contribution

Liyuan Han, Lina Zhang, Wenhua Xing, and Renjie Zhuo contributed equally to this work.

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Research Article

Cumulative Effects of Hypertension, Dyslipidemia, and Chronic Kidney Disease on Carotid Atherosclerosis in Chinese Patients with Type 2 Diabetes Mellitus

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Aims. The aim of this study is to determine the extent of carotid atherosclerosis in Chinese patients with type 2 diabetes in relation to the cumulative atherosclerosis risk factors using ultrasonography. *Methods.* The presence of hypertension, dyslipidemia, and chronic kidney disease (CKD) was documented in 106 Chinese subjects with type 2 diabetes. Subjects with 0, 1, and ≥2 additional atherosclerosis risk factors were assigned into groups 1, 2, and 3, respectively (n = 17, 49, and 40, resp.). Using ultrasound, the carotid arteries were assessed for the presence of carotid plaque, plaque score, intima-media thickness (IMT), and carotid arterial stiffness. *Results.* With the adjustment for age and gender, the presence of plaque and plaque score were significantly higher in groups with more atherosclerosis risk factors ($P < 0.05$). In addition, age > 60 years old (odds ratio = 2.75; 95% CI: 1.26–6.0) and the presence of hypertension (odds ratio = 2.48; 95% CI: 1.11–5.58), dyslipidemia (odds ratio = 2.41; 95% CI: 1.05–5.51), and CKD (odds ratio = 7.80; 95% CI: 1.46–41.72) could independently predict higher plaque score ($P < 0.05$). *Conclusions.* Hypertension, dyslipidemia, and CKD in Chinese patients with type 2 diabetes have cumulative effects on the burden of carotid plaque.

1. Introduction

Carotid atherosclerosis, a chronic disease with increased carotid arterial wall thickness as well as stiffness and/or the development of carotid plaque, is associated with the occurrence of cerebrovascular events, including transient ischemia attack (TIA) and stroke. According to the World Health Organization (WHO), about 15 million of people worldwide suffer from stroke annually, and one-third of them may die and another one-third may be permanently disabled.

Type 2 diabetes mellitus (DM) is one of the major risk factors associated with carotid atherosclerosis [1]. It has been suggested that patients with type 2 diabetes have thicker and stiffer carotid arteries and are more likely to suffer from cerebrovascular events [2, 3]. In addition, atherosclerotic process in the carotid artery also associates with different traditional atherosclerosis risk factors such as hypertension [4], dyslipidemia [4], and chronic kidney disease (CKD) [5]. Moreover, these proatherosclerotic diseases are common complications in patients with type 2 diabetes [6–8]. However, it is still unclear whether the clustering of these risk factors cumulatively increases the carotid atherosclerosis burden in patients with type 2 diabetes.

Plaque, IMT, and arterial stiffness of the carotid artery are common characteristics for assessing carotid atherosclerosis. Carotid plaque narrows the lumen of the carotid artery or may rupture leading to the formation of thrombus, reducing or blocking the blood supply to the brain. Carotid plaque score has been used to quantify the severity of carotid atherosclerosis [9]. In addition, carotid intima-media thickness (IMT) and carotid arterial stiffness are structural and functional parameters of the carotid artery, respectively, which have been suggested as indicators of early carotid atherosclerosis and predictors of future cerebrovascular and

cardiovascular events [10–12]. These atherosclerotic characteristics could be evaluated by ultrasonography. B-mode ultrasound is a noninvasive imaging method to assess carotid plaque and recent radiofrequency-based ultrasound technologies offer quantitative assessment of carotid IMT and carotid arterial stiffness [13, 14].

Thus, the present study aimed to investigate the cumulative effects of hypertension, dyslipidemia, and CKD on the characteristics of carotid atherosclerosis, including the presence of plaque, carotid plaque score, carotid IMT, and carotid arterial stiffness in Chinese patients with type 2 DM.

2. Materials and Methods

2.1. Subjects. In the study, subjects with type 2 diabetes were recruited from a local Chinese nonprofit making organisation for patients with diabetes (Angel of Diabetics Organisation, Hong Kong). The organisation has over 3000 registered patients who have been clinically diagnosed with DM and have regular follow-ups in diabetes clinics. Posters were put up in the premises of the Angel of Diabetics Organisation for recruitment of the patients. In the present study, the inclusion criteria of the subjects were Chinese patients with type 2 DM and older than 18 years, while the exclusion criteria of subjects were previous radiotherapy of the neck, carotid endarterectomy, and carotid stenting. Other atherosclerosis risk factors of subjects, including smoking, hypertension, dyslipidemia, and CKD, were identified with a questionnaire and a blood test. However, there was only one smoker in the 107 recruited subjects and was subsequently excluded to avoid statistical bias. As a result, a total of 106 Chinese subjects with type 2 diabetes were included in the study. The mean age of the subjects was 58.1 ± 9.0 years (ranging from 35 to 78 years) and 63.2% of them were women ($n = 67$).

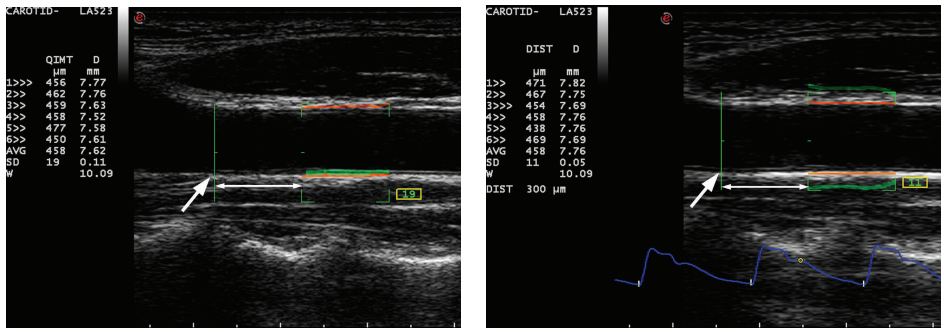
This study was approved by the Human Subject Ethics Subcommittee of the Hong Kong Polytechnic University. Written consent was obtained from the subjects before the commencement of the interview and ultrasound examination.

2.2. Identification of Atherosclerosis Risk Factors. For each subject, the brachial blood pressure was measured with a sphygmomanometer (Tensoval, Hartmann, Germany) at the left upper arm in sitting posture after at least 10 minutes of rest. A total of 3 mL of overnight fasting blood sample was obtained and blood tests were performed by a certified medical laboratory (Bright Growth Medical Laboratory Limited, Hong Kong). The levels of blood glucose, hemoglobin A1c (HbA1c), total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), triglyceride, and creatinine were determined by automated clinical chemistry analyzer using reagent cartridges recommended by the manufacturer (Dimension Xpand Plus, Siemens Healthcare, Germany). Estimated glomerular filtration rate (eGFR) was defined and calculated using the CKD-EPI (chronic kidney disease epidemiology collaboration) equation [15]. Subjects were interviewed and their medical history of coronary heart disease, stroke, smoking, and medical treatments of hypertension, dyslipidemia, and CKD was obtained. The presence of smoking, hypertension, dyslipidemia, and CKD was identified with the following criteria [16–18]: smoking: current smoker consuming 10 cigarettes per day for at least six months; hypertension: blood pressure ≥ 140/90 mmHg or under hypotensive medication; dyslipidemia: fasting total cholesterol ≥ 5.2 mmol/L, low density lipoprotein ≥ 3.4 mmol/L, high density lipoprotein ≤ 1.0 mmol/L, triglyceride ≥ 1.7 mmol/L or under medication to lower level of cholesterol; CKD: eGFR < 60 mL/min per 1.73 m^2 or with kidney damage (i.e., kidney failure).

2.3. Ultrasound Examinations. All ultrasound examinations were performed in a 22°C air-conditioned examination room using the Esaote MyLab Twice ultrasound unit in conjunction with a 4–13 MHz linear transducer (Esaote, Genoa, Italy). For each subject, the systolic and diastolic pressures were inputted into the ultrasound unit for evaluating carotid arterial stiffness.

Both the left and right carotid arteries were scanned and evaluated. All ultrasound examinations were performed by the same operator. The carotid arteries were assessed for the presence of carotid plaque, carotid plaque score, carotid IMT, and carotid arterial stiffness. Plaque was defined as focal thickening >50% of the adjacent intima-media layer [19]. When a plaque was identified, the plaque score was evaluated using a carotid plaque scoring system [9]. In the scoring system, the carotid artery was divided into five segments: (1) proximal common carotid (≥2 cm proximal to carotid bifurcation), (2) distal common carotid (<2 cm proximal to carotid bifurcation), (3) carotid bulb and bifurcation, (4) internal carotid artery, and (5) external carotid artery. In each segment, transverse B-mode images of the plaque were obtained and the degree of carotid artery stenosis was identified as the percentage reduction of lumen diameter at the most stenotic site. The plaque score was graded as follows: grade 0, normal or no detectable plaque; grade 1–5, at least one plaque with stenosis degree <30%, 30–49%, 50%–69%, 70%–99%, and 100%, respectively. The carotid plaque score of each subject was expressed as the summation of the scores of all segments in both carotid arteries.

Carotid IMT and carotid arterial stiffness were evaluated using the automated quantification programmes of the ultrasound unit: radiofrequency-based quality intima-media thickness (RF-QIMT) and radiofrequency-based quality arterial stiffness (RF-QAS), respectively (Figure 1) [14]. Carotid IMT was measured at the far wall of the common carotid artery (CCA) at a 1 cm segment 1 cm proximal to the inferior end of the carotid bulb. Carotid arterial stiffness was evaluated over the near and far walls of the CCA at the same segment (Figure 1). In the evaluation of carotid IMT and carotid arterial stiffness, the mean and standard deviation (SD) of the measurements in six consecutive cardiac cycles were automatically and continuously recorded by the system, and the mean measurement with a SD of <20 μm for IMT or <30 μm for stroke change in diameter for carotid arterial stiffness was obtained for data analyses. Each carotid artery



(a) (b)

Figure 1: Measurements of the intima-media thickness and stiffness in the common carotid artery. (a) Longitudinal grey scale sonogram showing the measurement of the intima-media thickness of a common carotid artery (CCA) using radiofrequency-based quality intima-media thickness. (b) Longitudinal grey scale sonogram showing the measurement of the arterial stiffness of the same CCA using radiofrequency-based quality arterial stiffness. The arrows indicate the inferior end of the carotid bulb and the double-arrows lines show a distance of 1 cm.

was scanned three times for measuring carotid IMT and carotid arterial stiffness.

In the evaluation of carotid arterial stiffness, five arterial stiffness parameters were investigated in the study: distensibility coefficient (DC), compliance coefficient (CC), the indices of α and β, and pulse wave velocity (PWV). The lower DC and CC and the higher α, β and PWV, the stiffer the carotid artery. The equations of these parameters are

$$\begin{aligned} \mathrm{DC} &= \frac{\Delta A/A_d}{\Delta P}\ (1/\mathrm{KPa}), \\ \mathrm{CC} &= \frac{\Delta A}{\Delta P}\ \left(\mathrm{mm}^2/\mathrm{KPa}\right), \\ \alpha &= \frac{A_d \cdot \ln(\mathrm{SBP}/\mathrm{DBP})}{A_s - A_d}, \\ \beta &= \frac{D_d \cdot \ln(\mathrm{SBP}/\mathrm{DBP})}{D_s - D_d}, \\ \mathrm{PWV}\ (\mathrm{m/s}) &= \sqrt{\frac{\alpha \cdot \mathrm{DBP}}{\rho}}, \end{aligned} \tag{1}$$

where D_s and D_d mean the systolic and diastolic diameters of the artery; A_s and A_d refer to the systolic and diastolic lumen areas and SBP and DBP indicate the systolic and diastolic blood pressures; ΔD, ΔA, and ΔP represent the stroke change in the diameter, the lumen area of the artery, and the blood pressure, respectively; and ρ is the blood density.

2.4. Statistical Analysis. Continuous data are expressed as means ± SD. The normality of distribution was checked using Shapiro-Wilk test. The adjusted comparisons between study groups were performed using ANCOVA or logistic regression. The association between atherosclerosis risk factors of subjects and carotid plaque score were determined using ordinal regression. Paired t-test and Wilcoxon signed ranks test were used to compare the measurements at the left and right carotid arteries of the same individuals. All statistical analyses were performed using SPSS 20 (IBM, Armonk, New York, United States) and P value <0.05 was considered as significant.

3. Results

3.1. The Effects of Atherosclerosis Risk Factors on Carotid Atherosclerosis in Chinese Subjects with Type 2 Diabetes. In the 106 subjects with type 2 diabetes, the mean blood glucose and HbA1c level were 7.53 ± 1.65 mmol/L and 6.90 ± 0.96%, respectively. Among these 106 subjects, 17 subjects did not have any additional atherosclerosis risk factor (Group 1), 49 had one additional atherosclerosis risk factor (Group 2), and 40 had two or three additional atherosclerosis risk factors (Group 3). There were no significant differences of blood glucose and HbA1c level between the three groups (Table 1; $P > 0.05$).

As shown in Table 1, the age- and gender-adjusted analyses demonstrated that there were significant differences of the presence of carotid plaque between groups 1, 2, and 3 ($P < 0.05$). Subjects in groups 1, 2, and 3 had the incidence of carotid plaque of 23.5% (4 of 17), 38.8% (19 of 49), and 60.0% (24 of 40), respectively (Table 1). Subjects with more atherosclerosis risk factors had higher possibility to have carotid plaque.

Similarly, ordinal logistic regression analysis with the adjustment of age and gender showed carotid plaque score was significantly higher in groups with more atherosclerosis risk factors (Table 1; $P < 0.05$). In addition, another ordinal logistic regression model indicated that the age > 60 years old and the presence of hypertension, dyslipidemia, and CKD led to higher plaque scores in the 106 subjects ($P < 0.05$, Table 2).

In contrast, with the adjustment of age and gender of the subjects (ANCOVA), no significant differences of carotid IMT and carotid arterial stiffness were found in groups with different numbers of atherosclerosis risk factors.

3.2. Carotid IMT and Carotid Arterial Stiffness in the Carotid Artery with or without Plaque. In this study, it was found that

Table 1: Demographic and ultrasonographic characteristics in Chinese subjects with type 2 diabetes with different numbers of atherosclerosis risk factors.

Parameters	Total	Groups with different number of atherosclerosis risk factor			P value (Adjusted)
		Group 1 0 (n = 17)	Group 2 1 (n = 49)	Group 3 ≥2 (n = 40)	
Age, years	58.1 ± 9.0	56.9 ± 7.8	57.1 ± 8.9	60.0 ± 9.4	—
Gender (female/male), n	67/39	11/6	27/22	29/11	—
Presence of plaque, n	47	4	19	24	0.017*
Plaque score	—	—	—	—	0.005*
Hypertension, n	60	—	27	33	—
Dyslipidemia, n	65	—	28	37	—
CKD, n	5	—	0	5	—
Coronary heart disease, n	1	0	0	1	—
Stroke, n	3	0	1	2	—
Blood glucose, mmol/L	7.53 ± 1.65	7.36 ± 1.41	7.63 ± 1.85	7.47 ± 1.51	0.921
HbA1c, %	6.90 ± 0.96	6.74 ± 0.82	6.98 ± 1.05	6.85 ± 0.91	0.981
Total cholesterol, mmol/L	4.72 ± 0.85	4.38 ± 0.53	4.73 ± 0.80	4.84 ± 0.99	0.080
HDL, mmol/L	1.33 ± 0.30	1.32 ± 0.12	1.34 ± 0.34	1.32 ± 0.31	0.814
LDL, mmol/L	2.82 ± 0.73	2.54 ± 0.48	2.86 ± 0.67	2.89 ± 0.86	0.124
Triglyceride, mmol/L	1.23 ± 0.72	1.12 ± 0.48	1.16 ± 0.68	1.36 ± 0.84	0.203
eGFR, mL/min per 1.73 m^2	87.81 ± 14.37	94.33 ± 12.67	88.66 ± 13.05	84.02 ± 15.70	0.022*
IMT, μm	685.1 ± 123.3	676.7 ± 119.5	671.5 ± 128.5	705.5 ± 118.6	0.624
DC, 1/KPa	0.017 ± 0.007	0.018 ± 0.005	0.017 ± 0.008	0.015 ± 0.005	0.441
CC, mm^2/KPa	0.781 ± 0.381	0.908 ± 0.719	0.762 ± 0.311	0.750 ± 0.280	0.343
α	5.590 ± 1.729	5.400 ± 1.739	5.550 ± 1.774	5.715 ± 1.703	0.866
β	11.302 ± 3.494	10.530 ± 3.445	11.301 ± 3.577	11.632 ± 3.446	0.633
PWV, m/s	7.986 ± 1.284	7.583 ± 1.237	7.990 ± 1.323	8.151 ± 1.250	0.434

* P value (Adjusted) indicates significant difference after the adjustment of age and gender. CKD: Chronic kidney disease; eGFR: estimated glomerular filtration rate, IMT: intima-media thickness; DC: distensibility coefficient; CC: compliance coefficient; and PWV: pulse wave velocity.

Table 2: The effect of atherosclerosis risk factors on carotid plaque score in subjects with type 2 diabetes (n = 106).

Parameters	Number of total	Odds ratio	Plaque score 95% CI		P value
			Lower	Upper	
Gender (No. of female)	67	1.22	0.55	2.71	0.634
Age (>60 years)	50	2.75	1.26	6.00	0.011*
Hypertension	60	2.48	1.11	5.58	0.027*
Dyslipidemia	65	2.41	1.05	5.51	0.037*
CKD	5	7.80	1.46	41.72	0.016*

* P value indicates significant difference. 95% CI: 95% confidence interval. CKD: chronic kidney disease.

the carotid artery with plaque did not have increased carotid IMT and carotid arterial stiffness. In the 106 subjects, 47 had carotid plaque while the other 59 subjects did not. After the adjustment of age and gender of subjects (ANCOVA), there were no significant differences of carotid IMT and carotid arterial stiffness in subjects with or without plaque (Table 3; $P > 0.05$). Furthermore, in the subjects with unilateral carotid plaque ($n = 29$), the IMT and arterial stiffness of the carotid artery with plaque were not significantly different from those of the contralateral carotid artery without plaque ($P > 0.05$).

3.3. The Left Carotid Artery versus the Right Carotid Artery. The results of the study suggested that the left carotid artery in subjects with type 2 diabetes was more vulnerable to atherosclerosis when compared with the right carotid artery. In the 106 subjects, there were greater carotid IMT and smaller DC (stiffer) in the left carotid artery than in the right

Table 3: Carotid IMT and carotid arterial stiffness in subjects with type 2 diabetes.

Parameters	Subjects with type 2 diabetes					
	The subjects with plaque ($n = 47$)	The subjects without plaque ($n = 59$)	P value (Adjusted)	The left carotid artery ($n = 106$)	The right carotid artery ($n = 106$)	P value
IMT, μm	706.6 ± 107.7	668.0 ± 132.8	0.171	703.3 ± 160.8	661.6 ± 129.5	<0.001*
DC, 1/KPa	0.016 ± 0.006	0.018 ± 0.007	0.758	0.016 ± 0.007	0.017 ± 0.010	0.031*
CC, mm^2/KPa	0.768 ± 0.262	0.800 ± 0.456	0.426	0.750 ± 0.438	0.777 ± 0.353	0.111
α	5.930 ± 1.665	5.319 ± 1.745	0.369	5.771 ± 2.225	5.553 ± 2.425	0.132
β	12.066 ± 3.359	10.694 ± 3.508	0.203	11.676 ± 4.444	11.274 ± 4.898	0.153
PWV, m/s	8.162 ± 1.276	7.845 ± 1.284	0.209	8.144 ± 1.612	8.014 ± 1.721	0.265

*P value (Adjusted) indicates significant difference after the adjustment of age and gender. *P value indicates significant difference. CKD: Chronic kidney disease; IMT: intima-media thickness; DC: distensibility coefficient; CC: compliance coefficient; and PWV: pulse wave velocity.

carotid artery (Table 3; $P < 0.05$). Similarly, plaque was more commonly found in the left carotid artery for subjects with unilateral carotid plaque (total: $n = 29$ and left: $n = 18$ or 62% *versus* right: $n = 11$ or 38%).

4. Discussion

DM, mainly type 2 DM, is an increasing health problem worldwide. It is estimated that global diabetes adults will achieve 552 million in 2030 [20]. In China, an epidemic study found that there were 92.4 million diabetes adults and 148.2 million prediabetes adults in 2010 [21], indicating that diabetes is a major public health problem in this country to date.

DM is a common cause of atherosclerosis [1], and patients with diabetes tend to suffer hypertension, dyslipidemia, and CKD, which are also classical risk factors of atherosclerosis [6–8]. The present study found that Chinese type 2 diabetics with more additional atherosclerosis risk factors had higher incidence of carotid plaque and higher carotid plaque score. In addition, in Chinese subjects with type 2 diabetes, gender did not have significant effects on carotid plaque score, whereas the age > 60 years old and the presence of hypertension, dyslipidemia, and CKD independently predicted higher carotid plaque score (Table 2; $P < 0.05$). Among these effective predictors, CKD showed the highest relative importance in the prediction of higher carotid plaque score (odds ratio = 7.80; 95% CI: 1.46–41.72), followed by age >60 years old (odds ratio = 2.75; 95% CI: 1.26–6.0), hypertension (odds ratio = 2.48; 95% CI: 1.11–5.58), and dyslipidemia (odds ratio = 2.41; 95% CI: 1.05–5.51). It was suggested that age > 60 years old and the presence of hypertension, dyslipidemia, and CKD additively increased the presence and the score of carotid plaque in Chinese patients with type 2 diabetes. Therefore, prompt diagnoses and appropriate treatments for these atherosclerosis risk factors are necessary in Chinese patients with type 2 diabetes particularly for elder patients, and the patients with more additional atherosclerosis risk factors need more concern for atherosclerosis of the carotid artery.

However, results showed that carotid IMT and carotid arterial stiffness did not significantly increase in the groups with more additional atherosclerosis risk factors (Table 1). This result is consistent with a previous study in which diabetes and hypertension did not have additive effect on carotid thickening and stiffening [2]. The result may be due to the fact that the CCA was used for the measurement of carotid IMT and carotid arterial stiffness. The IMT and stiffness of the CCA are commonly used to represent carotid IMT and carotid arterial stiffness, but they just reflect the conditions of local carotid arterial wall or the systemic arterial wall to some extent [2]. Compared with the carotid bifurcation and bulb, the CCA is a less susceptible site to atherosclerosis [22]. Hypertension, dyslipidemia, and CKD induce abnormal shearing pressure to the endothelium, greater circulating cholesterol level, and more severe oxidative stress in blood, respectively [1]. The CCA may be less influenced by these proatherosclerotic conditions, while the bifurcation and bulb may be more susceptible to these proatherosclerotic conditions and be easier to have atherosclerotic changes. Consequently, carotid plaque, which is predominantly found in the carotid bifurcation and bulb, may not imply thickening and stiffening of the CCA, and thus measurements in the CCA may not fully reflect atherosclerotic burdens in the carotid artery. This conclusion was also supported by other findings of the present study that there were no significant differences of carotid IMT and carotid arterial stiffness between subjects with and without carotid plaque; and in subjects with unilateral carotid plaque, the carotid artery with plaque was not significantly thicker and stiffer than the contralateral carotid artery without plaque. Thus, composite evaluations, including the presence of carotid plaque, carotid plaque score, carotid IMT, and carotid arterial stiffness, may be necessary in clinical researches and practices.

The present study found that there were different atherosclerosis burdens between the left and right carotid artery in Chinese subjects with type 2 diabetes; the left carotid artery was more susceptible to carotid atherosclerosis. The result was consistent with Luo's study in which the left carotid arterial wall was thicker than the right in the elderly as well as in the subjects with atherosclerotic diseases [23]. The asymmetry between the left and right carotid arteries may be due to the anatomy of the circulation system. Normally, the left carotid artery branches from the arch of aorta while

the right carotid artery branches from the innominate artery [24]. The different origins of the left and right carotid arteries induce different haemodynamics to the two arteries. If there are proatherosclerotic conditions, the asymmetric haemodynamics of the left and right carotid arteries may induce the development of carotid atherosclerosis at different degrees [23, 25]. Thus, the left carotid artery may need more concern, and carotid IMT and carotid arterial stiffness of the left carotid artery may be used to represent the atherosclerotic conditions of individuals with diabetes in future clinical researches or practices.

A limitation of this study is the small size of subjects, and thus the additive effects of atherosclerosis risk factors on carotid atherosclerosis in Chinese patients with type 2 diabetes may be not fully evaluated. Future investigations with larger sample size remain to be conducted. Because of limited number of subjects with smoking, this study did not assess the additive effect of smoking on carotid atherosclerosis in Chinese type 2 diabetics. Actually, smoking is a well-established risk factor of atherosclerosis and was shown to accentuate atherosclerosis in type 2 diabetics [26]. The only smoker in the present study also had 22% and 34% stenosis in the left and right carotid arteries, respectively. Future studies remain to be performed to comprehensively investigate the additive effect of smoking to carotid atherosclerosis in type 2 diabetics.

5. Conclusion

This study provides an understanding of the effects of additional atherosclerosis risk factors to carotid atherosclerosis in Chinese patients with type 2 DM. In these patients, the presence of hypertension, dyslipidemia, and CKD had cumulative effects on the burden of carotid plaque. As a result, prompt diagnoses and treatments of hypertension, dyslipidemia, and CKD are necessary for patients with diabetes, and more concern of carotid atherosclerosis should be given to the patients with more additional atherosclerosis risk factors.

Conflict of Interests

All authors declare no conflict of interests for the data, results, and conclusions described in this study.

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Research Article

Serum Fibroblast Growth Factor 21 Levels Are Correlated with the Severity of Diabetic Retinopathy

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The aim of the study was to assess serum fibroblast growth factor 21 (FGF21) concentrations in Chinese type 2 diabetic patients with and without retinopathy and to assess the association between FGF21 and the severity of retinopathy. 117 diabetic patients were compared with 68 healthy controls. Fasting blood glucose, serum total cholesterol, serum triglycerides, serum insulin, and serum FGF21 levels were estimated. FGF21 concentrations in the patients were significantly higher than those in control. In the patient group there was a significant positive correlation between FGF21, insulin level, and homeostasis model assessment index. Serum FGF21 concentrations in patients with proliferative diabetic retinopathy or nonproliferative diabetic retinopathy were significantly higher than those in patients without diabetic retinopathy. When the presence of diabetes was defined as the final variable in the conditional logistic regression model with the FGF21 concentration as the continuous variable, FGF21 was significantly involved in the model. This study shows that the increase in serum concentration of FGF21 was associated with the severity of diabetic retinopathy and suggests that FGF21 may play a role in the pathogenesis of diabetic retinopathy and its degree.

1. Introduction

Diabetes mellitus continues to be a tremendous burden throughout the world and is a significant cause of morbidity and mortality. Diabetic retinopathy is a microvascular complication of diabetes correlated with chronic hyperglycemia [1]. Diabetic retinal neovascularization is considered to be a major consequence of retinal ischemia caused by capillary occlusion, and the mechanism of its development is not clear [2, 3]. Diabetic retinopathy progresses from mild nonproliferative abnormalities (characterized by increased vascular permeability) to moderate and severe nonproliferative diabetic retinopathy (NPDR) (characterized by vascular closure) and then to proliferative diabetic retinopathy (PDR) (characterized by the growth of new blood vessels on the retina and posterior surface of the vitreous) [4, 5]. Macular edema (characterized by retinal thickening from leaky blood vessels) can be developed at any stage of retinopathy. The new blood vessels of PDR and contraction of all accompanying fibrous tissues can distort the retina and lead to fractional retinal detachment, producing severe and irreversible vision loss [6].

Despite improvements in glycemic control, the incidence of blindness in diabetic patients has progressively increased. Pharmacological and nonpharmacological interventions that target growth factor pathways are currently being investigated as a potential approach for the prevention and treatment of diabetic retinopathy [7, 8]. Many growth factors are implicated in the development of vascular pathologies in diabetic retinopathy, including vascular endothelial growth factor

(VEGF), platelet derived growth factor (PDGF), insulin-like growth factor (IGF), and fibroblast growth factor (FGF) [9–12].

The role of these growth factors in the pathogenesis of diabetic retinopathy is complex. The fibroblast growth factor family is composed of 22 members with a wide range of biological functions, including cell growth, development, angiogenesis, and wound healing [13]. FGF21 is a member of the endocrine FGF subfamily, which is expressed predominantly in the liver and stimulates glucose uptake through the induction of GLUT1 in adipocytes [14]. *In vivo* treatment with FGF21 results in the amelioration of glucose and regulates lipid metabolism in both murine and nonhuman primate models of diabetes and obesity [15]. Taken together, these findings demonstrate that FGF21 plays an important role in the regulation of glucose and lipid metabolism and also suggest that FGF21 exhibits the therapeutic characteristic necessary for the effective treatment of diabetes and obesity.

Recent human studies indicated that increased serum levels of FGF21 are found in obese individuals and subjects with metabolic syndrome and type 2 diabetes mellitus [16–18]. Whether FGF21 is closely associated with the pathology of retinopathy in diabetic patients remains unclear. To explore the physiological and pathological relevance of FGF21 in patients with diabetic retinopathy, we measured the serum concentrations of FGF21 in Chinese subjects and analyzed its association with a cluster of metabolic parameters that related to the changes seen in retinopathy.

2. Methods

2.1. Study Subjects. The study population consisted of 117 outpatients with diabetic retinopathy and 68 healthy individuals. The 117 patients with diabetic retinopathy were recruited from outpatient ophthalmology clinics at Xiamen Hospital of Traditional Chinese Medicine, Xiamen. Patients recruited into this study were aged ≥25 between October 8, 2009, and May 11, 2012. Patients who were seen in the retina, glaucoma, cornea, and comprehensive ophthalmology clinics during the enrollment period were considered potential study subjects. After undergoing routine ophthalmic examination, subjects were recruited and divided into the four groups, based on their diabetes status and retinopathy findings. The control group is the no diabetic retinopathy (no DR) group consisting of subjects with type 2 diabetes but no evidence of diabetic retinopathy, such as microaneurysms, cotton-wool spots, intraretinal hemorrhages, or macular edema. The nonproliferative diabetic retinopathy (NPDR) group comprised patients with evidence of retinopathy such as microaneurysms, cotton-wool spots, intraretinal hemorrhages, or macular edema but no evidence of retinal or iris neovascularization. Lastly, the proliferative diabetic retinopathy (PDR) group consisted of subjects with neovascularization on the optic disc, retina, or iris, with or without vitreous hemorrhage or prior panretinal photocoagulation. When the diabetic retinopathy was asymmetric, the subject was assigned to the group according to the eye with the worst retinopathy. Subjects were excluded if they had type 1 diabetes, were younger than 18, or were older than 90 years of age.

Sixty-eight healthy volunteers served as the control group. They were matched with the diabetic patients according to age, BMI, blood pressure, and sex. They underwent routine physical and laboratory evaluations to ensure that none had diabetes or other metabolic, hepatic, or renal diseases. In addition, none of the healthy volunteers had a family history of hypertension or diabetes. Subjects in this group were not excluded if they had other forms of ocular disease, such as uveitis or macular degeneration. All individuals gave informed consent to participate in the study, which was approved by the Ethics Committee of Fujian Traditional Chinese Medicine University.

2.2. Biochemical Measurements. Once enrolled in the study, subjects completed a medical history questionnaire and had their blood drawn. Patients were not required to fast prior to this blood draw. One blood sample was immediately tested for Glycated hemoglobin (HbA1c). Other whole blood, serum, and plasma samples were frozen at −80°C. Serum concentrations of glucose (glucose oxidase peroxidase calorimetric method) and creatinine (Jaffe' method) were determined using a Technicon Dax-48 system analyzer (Miles, Tarrytown, NY, USA). HbA1c was determined by inhibition of latex agglutination, using a DCA 2000 analyzer (Bayer, Elkhart, IN, USA). Microalbuminuria was detected by an immunoturbidimetric method with an Urinpak Micro Albumin immunokit (Miles, Tarrytown, NY, USA). Total cholesterol, high-density lipoprotein cholesterol, and triglyceride levels were determined by an enzymatic colorimetric method with an Olympus AU 600 autoanalyzer, using reagents from Olympus Diagnostics GmbH (Hamburg, Germany). Low-density lipoprotein (LDL) cholesterol level was calculated by Friedewald's formula.

The basal serum concentration of insulin was determined by the coated-tube method (Diagnostic Products Corporation, Los Angeles, CA, USA). In brief, a homeostasis model of assessment insulin resistance score (HOMA-IR) was computed with the following formula: (HOMA-IR) = fasting plasma glucose (mg/dL) × immunoreactive insulin (μU/mL)/405 [19]. Serum levels of FGF-21 (Biovendor, Modrice, Czech Republic) and C-reactive protein (CRP, R&D, USA) were determined with commercially available enzyme-linked immunosorbent assays according to the manufacturers' instructions.

2.3. Statistical Analysis. Data were processed using the SPSS 11.5 statistical package. Results are reported as mean ± SD. Levene's test was used to evaluate the distribution characteristics of the variables. Differences between diabetic and control groups were tested for significance by t-tests, Mann-Whitney U tests and χ^2-tests. The relationship between variables was analyzed by Pearson's correlation. The results were also analyzed by one-way analysis of variance, Bonferroni-adjusted Mann-Whitney U tests, and t-tests for the comparison of subgroups. We used the odds ratio (OR) as a measure of association between outcome and exposure and estimated the adjusted odds ratios in conditional logistic regression analyses.

Table 1: Clinical and laboratory features of the patient and control groups. Values are means (±SD) or number (%).

	Control ($n = 68$)	Patients ($n = 117$)	P value
Age (years)	58.5 ± 11.3	60.3 ± 10.2	0.343
Sex (M/F)	37/31	63/54	0.362
BMI (kg/m^2)	25.7 ± 6.8	32.9 ± 7.9	<0.001
Systolic BP (mmHg)	129.88 ± 11.58	133.69 ± 8.32	0.462
Diastolic BP (mmHg)	83.63 ± 3.69	84.33 ± 4.39	0.653
Total cholesterol (mmol/L)	11.55 ± 2.13	11.39 ± 1.57	0.852
Triglycerides (mmol/L)	6.93 ± 1.74	7.21 ± 1.14	0.578
LDL cholesterol (mmol/L)	6.77 ± 3.61	6.54 ± 0.75	0.842
HDL cholesterol (mmol/L)	2.33 ± 0.58	2.31 ± 0.55	0.673
Insulin (μU/mL)	6.55 ± 1.03	13.13 ± 8.74	<0.001
HOMA-IR	1.33 ± 0.34	3.11 ± 0.84	<0.001
HbA1c (%)	5.9 ± 0.7	8.0 ± 0.8	<0.001
FPG (mmol/L)	4.54 ± 1.53	7.66 ± 1.47	<0.001
Macrovascular disease	13 (13%)	39 (36%)	<0.001
FGF21* (pg/mL)	125.9 ± 39.3	542.3 ± 80.5	<0.001

DM: diabetes mellitus, DR: diabetic retinopathy, NPDR: nonproliferative diabetic retinopathy, PDR: proliferative diabetic retinopathy, BP: blood pressure, and FPG: fasting plasma glucose. Macrovascular disease represents coronary disease, cerebrovascular disease, and peripheral vascular disease.
*Bonferroni-adjusted t-test: $P = 0.001$ compare with Control.

Receiver operating characteristics (ROC) curve analysis was performed to determine a threshold concentration of FGF21 for the development of diabetic retinopathy. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Subject Characteristics. The clinical and laboratory data of all participants are shown in Table 1. No significant differences in age and systolic and diastolic blood pressures were observed between the patient and control group. The mean body mass index (BMI) was statistically lower in the control group than the patient groups. The insulin, fasting blood glucose, and HOMA-IR indexes of the patients were significantly greater, while the FGF21 concentrations were also higher than those of the controls ($P < 0.001$ for all). The percentage of subjects with macrovascular disease was significantly lower in the no DM group, compared to the no DR, NPDR, and PDR groups.

There was a significant positive correlation between serum FGF21 and insulin or HOMA-IR index in the group of patients ($r = 0.498$, $P < 0.001$, and $r = 0.341$, $P < 0.05$, resp.). In addition, serum FGF-21 levels correlated with fasting glucose, fasting insulin, and HOMA-IR after adjustment for BMI, but no significant correlation was found between serum FGF-21 levels and lipid parameters, HOMA-IS. Furthermore, serum FGF-21 levels correlated with macrovascular disease, but the significant differences between the FGF21 concentrations of the subgroups were not affected by the sex distribution.

3.2. FGF-21 Analysis. After the patients were subdivided according to severity of retinopathy, age, sex, and concentrations of fasting blood glucose, HbA1c, and insulin, the HOMA-IR indexes were similar for each subgroup (Table 2). Serum FGF21 concentrations in patients with PDR ($n = 49$; 669.4 ± 89.2 pg/mL) or NPDR ($n = 34$; 631.9 ± 73.8 pg/mL) were significantly higher than those in patients without retinopathy ($n = 34$; 326.8 ± 81.6, $P < 0.001$). Serum FGF21 concentrations in the patients with PDR were not significantly higher than those in patients with NPDR ($P = 0.671$; Table 2). There was a significant positive correlation between serum FGF-21 levels and macrovascular disease in no DR, NPDR, and PDR patients. The effects of serum FGF21 concentrations on diabetes and diabetic retinopathy were assessed by conditional logistic regression analysis, after adjustment for the other parameters (HOMA-IR, insulin, HbA1c, FGF21, and glucose) by matching. When the presence of diabetes was defined as the final variable in the conditional logistic regression model with the FGF21 concentration as the continuous variable, FGF21 was significantly involved in the model ($P = 0.001$; Table 3). ROC curve analysis was performed in order to establish a threshold FGF21 concentration for the existence of diabetic retinopathy. However, there was not a statistically significant cut-off value of serum FGF21 for the condition (Figure 1).

4. Discussion

Diabetic retinopathy is the major cause of irreversible blindness in patients of working age throughout the world [20]. Diabetes reduces angiogenic potential in the retinal microvasculature, suggesting that, as well as inactive proliferative retinopathy, the angiogenic potential of retinal microvascular endothelial cells is significantly compromised by the diabetic state [21]. In China, through early detection of the condition through screening programs, patients who receive treatment are less likely to become blind from proliferative changes and maculopathy. However, a screening

Table 2: Clinical and laboratory features of type 2 diabetic patients with and without retinopathy. Values are means (±SD) or number (%).

	NO DR ($n = 34$)	NPDR ($n = 34$)	PDR ($n = 49$)	P value
Age (years)	59.4 ± 10.2	61.3 ± 10.1	59.2 ± 12.7	0.716
Sex (M/F)	20/14	22/12	21/28	0.964
Systolic BP (mmHg)	134.29 ± 12.41	129.77 ± 9.18	131.78 ± 6.70	0.268
Diastolic BP (mmHg)	85.77 ± 3.91	81.78 ± 2.47	83.68 ± 2.35	0.219
Total cholesterol (mmol/L)	11.55 ± 1.61	11.66 ± 1.23	10.79 ± 2.15	0.254
Triglycerides (mmol/L)	7.32 ± 1.32	7.49 ± 0.81	7.15 ± 1.56	0.288
LDL cholesterol (mmol/L)	6.63 ± 0.89	6.46 ± 0.79	6.31 ± 0.61	0.421
HDL cholesterol (mmol/L)	2.21 ± 0.44	2.31 ± 0.57	2.39 ± 0.77	0.216
BMI (kg/m^2)	32.3 ± 9.1	32.7 ± 7.3	33.1 ± 5.9	0.684
Insulin (μU/mL)	10.70 ± 3.13	12.08 ± 4.18	16.47 ± 4.59	0.076
HOMA-IR	2.96 ± 0.77	3.18 ± 0.60	3.25 ± 1.13	0.116
HbA1c (%)	7.7 ± 1.2	8.1 ± 0.3	8.3 ± 1.7	0.638
FPG (mmol/L)	7.52 ± 1.23	7.78 ± 1.38	7.68 ± 1.27	0.087
Macrovascular disease	6 (14%)	14 (33%)	19 (44%)	<0.001
FGF21* (pg/mL)	326.8 ± 81.6$^{*\#}$	631.9 ± 73.8†	669.4 ± 89.2	<0.001

Bonferroni-adjusted t-test: $^{*}P = 0.01$ compared with nonproliferative DR (NPDR), $^{\#}P = 0.001$ compared with proliferative DR (PDR), and $^{\dagger}P = 0.671$ compared with PDR.

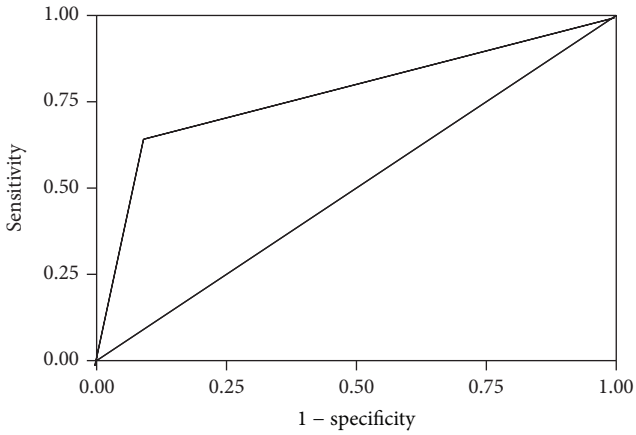

Figure 1: ROC curve of serum FGF21 concentrations for occurrence of diabetic retinopathy. The estimated cut-off value of FGF21 is 550 pg/mL, with 86.5% sensitivity and 75% specificity for the existence of diabetic retinopathy (area under the curve = 0.776, $P > 0.05$). Diagonal segments were produced by ties.

Table 3: Odds ratios (OR) and 95% confidence intervals (CI) for associations of FGF21 with diabetes ($n = 117$) and retinopathy ($n = 83$).

	OR (95% CI)
Diabetes	0.71 (0.62 to 0.81)
Retinopathy	0.73 (0.64 to 0.85)

program is effective only if patients at risk can be identified and persuaded to attend. Therefore, a reliable, easily performable, and inexpensive screening method is urgently required for the early detection of diabetic retinopathy [22]. The association of FGF21 with the severity of diabetic retinopathy may open up future projects on the early detection and prevention of the condition.

FGF21 is a unique member of the FGF family and plays a significant role in regulating glucose and lipid metabolism, including stimulating glucose uptake insulin-independently, improving hyperglycemia and dyslipidemia [15]. This study shows that serum FGF21 concentrations are higher in diabetic patients compared to controls and are higher in diabetic patients with DR than those without DR, suggesting that serum FGF21 may play a role in the pathogenesis of DR. In the present study, our data also indicate that serum FGF21 levels are significantly associated with insulin, HOMA-IR index, or macrovascular disease in NPDR and PDR subjects, suggesting that elevated FGF21 levels are closely related to the injury of capillary and the change of retinal function in diabetic retinopathy patients. However, the mechanisms responsible for the elevation of FGF21 concentration with the progression of diabetic retinopathy are not fully understood. FGF21 is expressed predominantly in liver and adipose tissue [23] and plays an important role in regulating lipid and energy metabolism [24, 25]. We speculate that the paradoxical increase in diabetic retinopathy patients is a compensatory mechanism to counteract metabolic stress. FGF21 resistance had been found in obesity and in cardiovascular failure, leading to compensatory upregulation of adiponectin [26, 27]. Based on these findings, we propose that the mechanism of increased FGF21 levels in diabetic retinopathy is similar to those observed in hyperglycemia-associated resistance to adiponectin. In response to endothelial dysfunction, serum adiponectin or FGF21, both as beneficial hormones to diabetes, may be compensatorily increased to repair microvascular legions involved in retinopathy.

The major finding in the present study was that serum concentrations of FGF21 were increased in diabetic patients but nonproportional to the severity of retinopathy. Although many factors are reported to affect the progression of diabetic

retinopathy [28–30], there is general agreement that the duration of diabetes and the severity of hyperglycemia are the major risk factors [8]. Thus, intensive treatment only results in delaying the procession of diabetic retinopathy and cannot prevent its development completely [31]. From the findings of the Diabetes Control and Complications Trial [22], susceptibility to diabetic retinopathy is influenced by genetic factors, in addition to other well-known causes of the condition. In recruiting the patients for this study, we were careful to ensure that all subgroups were similar, in terms of their body weight and other factors known to contribute to the development of retinopathy, such as HbA1c, time elapsed since diagnosis of diabetes, and coexistence of other significant health problems. The positive relationship between FGF21 concentration and retinopathy observed in this study was not accompanied by any obvious difference between the retinopathy subgroups with respect to factors promoting microangiopathy. According to the results of conditional logistic regression analysis, FGF21 is likely to be one of the major contributors to the pathogenesis of both type 2 diabetes and diabetic retinopathy. The results of the ROC curve analysis did not provide a threshold concentration of FGF21 for the existence of diabetic retinopathy and thus a predictive value cannot be assigned to FGF21 concentrations in relation to the development of the condition. This supposition therefore requires investigation in much larger sample groups.

However, there is no doubt that the limitations of FGF21 in diabetic retinopathy still need further study and investigation. The number of subjects in the study population was barely adequate to permit clear estimations to be made about the association of serum FGF21 concentrations with the severity of the diabetic retinopathy. It is like that with larger numbers of patients it would be possible to obtain a threshold for FGF21 concentration that is significant for the development of diabetic retinopathy. In addition, as a case-control design was used for the study used, it is not easy to accurately predict whether the high FGF21 concentrations preceded the retinopathy or vice versa. Future cohort studies will help provide more information on this.

In summary, the results from the present study suggest that serum FGF21 concentrations are higher in patients with type 2 diabetes than in age-, BMI-, and sex-matched controls. These concentrations are higher in patients with diabetic retinopathy than in those without it and are involved in the generation of diabetes and diabetic retinopathy. Future prospective studies with greater numbers of patients are recommended to establish a direct relationship between serum FGF21 concentrations and the severity of vascular complications.

Conflict of Interests

The authors confirm that the content of this paper has no conflict to interests.

Authors' Contribution

Yuan Lin, Ye-cheng Xiao, and Hong Zhu contributed equally to this work.

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Research Article

Evaluation of Serum Fibrinogen, Plasminogen, α2-Anti-Plasmin, and Plasminogen Activator Inhibitor Levels (PAI) and Their Correlation with Presence of Retinopathy in Patients with Type 1 DM

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Background. Diabetic retinopathy (DR) is the leading cause of blindness in the world. Retinopathy can still progress despite optimal metabolic control. The aim of the study was to determine whether different degrees of DR (proliferative or nonproliferative) were associated with abnormally modulated hemostatic parameters in patients with T1DM. *Method.* 52 T1DM patients and 40 healthy controls were enrolled in the study. Patients were subdivided into three categories. Group I was defined as those without retinopathy, group II with NPRP, and group III with PRP. We compared these subgroups with each other and the control group (Group IV) according to the serum fibrinogen, plasminogen, alpha2-anti-plasmin (α2-anti-plasmin), and PAI. *Results.* We detected that PAI-1, serum fibrinogen, and plasminogen levels were similar between the diabetic and control groups ($P = 0.209$, $P = 0.224$, and $P = 0.244$, resp.), whereas α2-anti-plasmin was higher in Groups I, II, and III compared to the control group ($P < 0.01$, $P < 0.05$, and $P < 0.001$, resp.). There was a positive correlation between serum α2-anti-plasmin and HbA1c levels ($r = 0,268$, $P = 0.031$). *Conclusion.* To our knowledge there is scarce data in the literature about α2-anti-plasmin levels in type 1 diabetes. A positive correlation between α2-anti-plasmin with HbA1c suggests that fibrinolytic markers may improve with disease regulation and better glycemic control.

1. Introduction

Diabetes mellitus (DM) is a condition associated with propensity for thrombosis and increased risk of cardiovascular complications, leading to morbidity and mortality of diabetic patients [1]. Microvascular and large vessel diseases are distinct in regard to pathophysiology, and there is no current evidence to suggest that these two conditions share a common pathogenetic pathway. Diabetic retinopathy (DR) is the leading cause of loss of vision in the world and of new cases of blindness in patients aged between 20 and 64 years [1].

Although strict glycemic control and the regulation of blood pressure reduce microvascular complications, retinopathy and nephropathy can still progress in some patients despite optimal metabolic control. This suggests that factors other than hyperglycemia, such as abnormal hemostatic parameters, may play a role in the disease pathogenesis. Based on this hypothesis, several markers of hypercoagulation including fibrinogen, plasma activator inhibitor

(PAI), and alpha-2-anti-plasmin have been identified. The hypercoagulable state can be explained by imbalance between levels of plasma procoagulant proteins and anticoagulant activity [2].

A positive association between increased levels of fibrinogen and the occurrence of microangiopathy was suggested. [3]. Myrup et al. demonstrated that fibrinogen levels were significantly higher in patients with nephropathy compared to those without [4]. Additional studies revealed that plasminogen and plasma activator inhibitor (PAI) levels were not affected in type 1 DM [5, 6], whereas coagulant activity was higher in patients with documented microvascular complications [7, 8]. Alpha 2-anti-plasmin (α2-anti-plasmin, or plasmin inhibitor) belongs to serine protease inhibitor (Serpin) family and it is responsible for inactivation of plasmin that is an important enzyme responsible for fibrinolysis and the degradation of various other proteins [9]. It was recently shown that there is a correlation between plasma plasmin-α2-anti-plasmin complex (PAP) and diabetic retinopathy regardless of other traditional risk factors for diabetic retinopathy, such as duration of diabetes, SBP, A1C, and serum creatinine levels [10].

The aim of the present study was to determine whether different degrees of DR (proliferative or nonproliferative) were associated with abnormally altered hemostasis in patients with type I DM. In addition, we planned to define whether there was a significant correlation between thrombotic tendency (as assessed by the levels of fibrinogen, PAI, plasminogen, and α2-anti-plasmin) and metabolic control.

Those parameters were compared in patients without retinopathy or with different degrees of retinopathy and the control group. To our knowledge, this is the first study that includes all these parameters and compares them between three different subgroups of type 1 diabetes patients containing relatively large numbers of patients.

2. Methods

2.1. Study Design. Fifty-two patients with type 1 diabetes (30 female and 22 male, with an age range of 17–63) who regularly visited our Endocrinology Clinics between January 2010 and December 2011 were enrolled in the present study. Each patient accepted and signed informed consent forms. Our local ethics committee approved the study. Medical history, current cigarette smoking, current alcohol consumption and drug history were recorded. Doppler analysis of vessels was used to detect the presence of macroangiopathy and venous or arterial thrombosis. Hypertension was defined as systolic blood pressure (SBP) > 140 mmHg, diastolic blood pressure > 90 mmHg, or the current use of antihypertensive medications. Resting blood pressure was measured three times in the seated position.

Patient selection was made by the same endocrinologist on the basis of the following inclusion criteria: type 1 DM (as defined by deficient c peptide secretion and autoantibody positivity at the time of diagnosis), duration of disease being longer than 5 years, normal blood pressure, normal serum triglyceride and LDL levels, and the absence of chronic kidney disease or peripheral obliterating arteriopathies, and other macrovascular disease. Forty healthy, nondiabetic subjects (20 male and 20 female), aged between 26 and 51 years, were recruited from hospital staff to serve as the control group.

Nephropathy was diagnosed by the presence of microalbuminuria (30–300 mg/day, Roche Diagnostics) or macroalbuminuria (>300 mg/day, Roche Diagnostics) in at least two samples of early morning urine collected over a 6-month period in the absence of infection, renal disease, or heart failure. Blood pressure and serum creatinine levels were measured to evaluate renal function.

All patients were treated only with subcutaneous insulin (human or analog). All test subjects were nonsmokers who had not taken drugs that could affect hemostasis for at least 4 weeks prior to the study.

2.2. Ocular Evaluation. All subjects underwent detailed ophthalmologic examination, including best-corrected visual acuity, applanation tonometry, anterior segment slit lamp biomicroscopy, and dilated fundus examination. Colored fundus photographs were taken from all subjects by nonmydriatic retinal camera (CR2-45 NM Nonmydriatic Fundus Camera, Canon Inc, Japan), and patients were then subdivided into three categories. Group I was defined as those without retinopathy, group II with nonproliferative retinopathy, and group III with proliferative retinopathy. Nonproliferative retinopathy (NPDR) was defined as nerve-fiber layer infarcts (cotton wool spots), intraretinal hemorrhages, hard exudates, and microvascular abnormalities (including microaneurysms, occluded vessels, and dilated or tortuous vessels) primarily in the macula and posterior retina. Proliferative diabetic retinopathy (PDR) was determined by the presence of neovascularization arising from the disc and/or retinal vessels and the resulting preretinal and vitreous hemorrhage, subsequent fibrosis, and traction retinal detachment.

2.3. Biochemical Evaluation. Blood samples were drawn into plain vacutainers from the antecubital veins of healthy controls and patients after 12 hours of fasting and 30 min of supine rest between 8 and 9 a.m. Glycated hemoglobin (HbA1c) was measured by high-performance liquid chromatography (HPLC), and the nondiabetic range used was 4.0–6.0%. To measure serum glucose and lipids (total cholesterol, triglycerides, and high density lipoprotein cholesterol), blood was drawn into evacuated siliconised tubes, and measurements were made by an autoanalyzer using enzymatic methods. The levels of plasma plasminogen, α2-anti-plasmin (Cusabio Biotech Co., Ltd), fibrinogen (MTI Tokra), and PAI-1 (Bender Med Systems, Vienna, Austria) were also measured.

2.4. Statistical Analysis. The SPSS version 11.5 software, Windows Edition (SPSS, Chicago, IL), was used for all statistical analysis. The nonparametric Kolmogorov-Smirnov test was used to compare samples with the reference probability distribution, whereas the homogeneity of the variances was

Table 1: Demographic data of the participants.

Variables	Group I (n: 21)	Group II (n: 18)	Group III (n: 13)	Group IV (n: 40)	P value
Age (years)	27.3 ± 7.4[a,b]	31.3 ± 9.9	40.2 ± 12.1[a]	35.6 ± 7.7[b]	**<0.001**
Sex					0.391
Female	10 (47.6%)	13 (72.2%)	7 (53.8%)	20 (50.0%)	
Male	11 (52.4%)	5 (27.8%)	6 (46.2%)	20 (50.0%)	
Duration of DM (years)	7 (5–20)[a]	8 (5–32)[c]	21 (5–40)[a,c]	—	**0.002**

[a]Difference between Group I and Group III is significant ($P < 0.001$). [b]Difference between Group I and Group IV is significant ($P = 0.006$). [c]Difference between Group II and Group III is statistically significant ($P < 0.001$).

Table 2: Biochemical and hemostatic measurements of diabetic patients and healthy controls.

Variables	Group I (n: 21)	Group II (n: 18)	Group III (n: 13)	Group IV (n: 40)	P value
HbA1c	9.2 (6.6–11.0)[a]	9.2 (6.0–11.0)[b]	8.5 (5.6–12.0)[c]	5.3 (4.4–6.3)[a,b,c]	**<0.001**
PAI	182.7 (9.4–271.0)	158.0 (7.9–255.2)	162.1 (7.9–246.7)	155.6 (107.2–236.9)	0.209
α2anti-plasmin	245.0 (31.0–650.0)[a]	202.0 (55.0–904.0)[b]	418.0 (42.0–1184.0)[c]	115.5 (23.0–591.0)[a,b,c]	**0.004**
Plasminogen	90.0 (69.0–152.0)	102.0 (55.0–141.0)	107.0 (57.0–133.0)	87.0 (68.0–121.0)	0.244
Fibrinogen	259.8 ± 72.6	277.3 ± 62.8	307.1 ± 81.3	272.1 ± 42.3	0.224

[a]Difference between Group I and Group IV is significant ($P < 0.01$). [b]Difference between Group II and Group IV is significant ($P < 0.05$). [c]Difference between Group II and Group IV is significant ($P < 0.001$).

assessed using Levene's test. Descriptive statistics are presented as means ± standard deviation (SD) and medians (minimum-maximum) for continuous variables and as percentages (%) for categorical variables. The significance of the differences between mean values was assessed by one-way ANOVA, while the Kruskal-Wallis test was used for median values. If significant differences were detected after one-way ANOVA or Kruskal-Wallis tests, post hoc Tukey's HSD or Conover's nonparametric multivariance comparison tests were used to define the effective factors. For categorical variables, differences were assessed by chisquared and Fisher's exact tests, as appropriate. A value of $P < 0.05$ was considered to indicate statistical significance.

3. Results

There were 21, 18, and 13 patients with type 1 diabetes in Group I, II, and III, respectively, while there were 40 individuals in the control group. All groups had similar gender distribution. Diabetic retinopathy was detected in 59.6% of the diabetic patients. The mean age of patients in group III (diabetics with PDR) was significantly higher than group I (diabetics without retinopathy). The age distribution of the control subjects was similar to the diabetic groups, except for group I, where the mean age was significantly less. The duration of diabetes was significantly longer in group III compared with group I and II ($P < 0.001$). However, no difference was detected between groups I and II (Table 1).

Microalbuminuria was detected in 12 patients (23.07%) and was predominantly associated with proliferative DR ($n = 7$) but was also observed in patients without retinopathy ($n = 1$) or with nonproliferative retinopathy ($n = 4$). In contrast, macroalbuminuria was observed only in patients with proliferative DR ($n = 4$; 7.67% of the diabetic population).

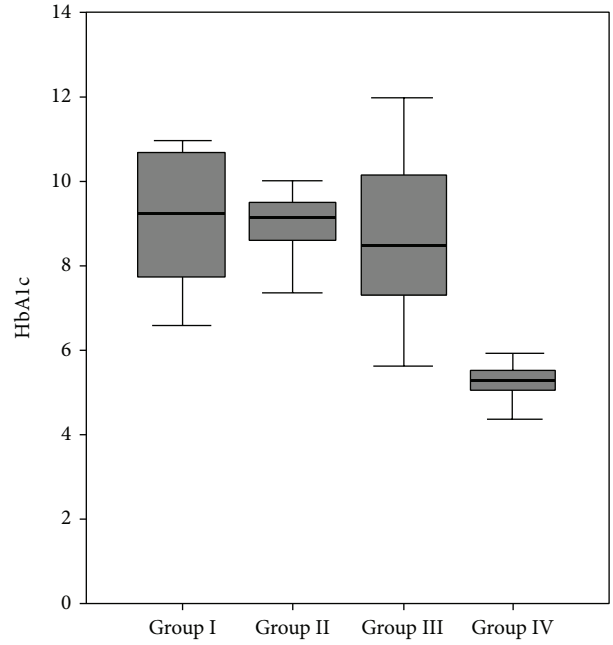

Figure 1: Distribution of HbA1c within the groups.

The median serum HbA1c levels were similar in the diabetic groups (group I, II, and III) and were increased significantly, compared with the control group, as expected ($P < 0.001$; Figure 1).

Serum PAI-1 levels were higher in the diabetic groups than control, but this was not statistically significant ($P = 0.209$). The levels of serum fibrinogen ($P = 0.224$) and plasminogen ($P = 0.244$) were similar between the diabetic and control groups (Table 2).

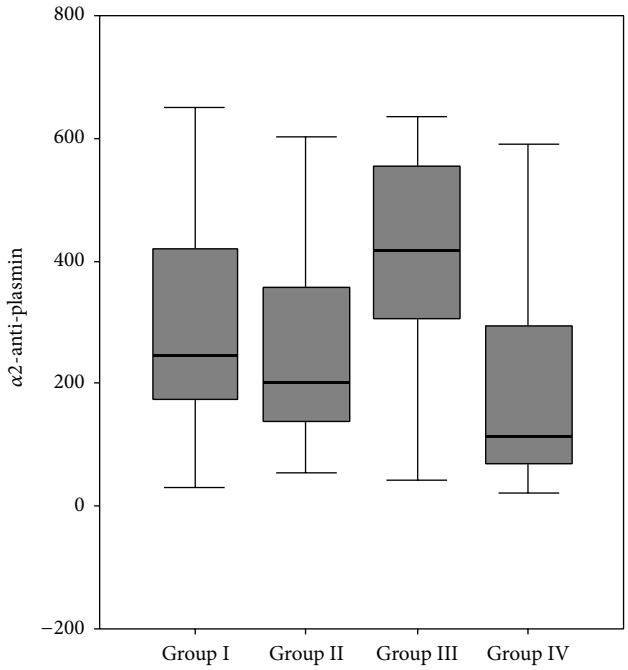

Figure 2: Expression of α2-anti-plasmin in the diabetic and control groups.

The levels of alpha-2-anti-plasmin were significantly higher in groups I, II, and III than the control group ($P < 0.01$, $P < 0.05$, and $P < 0.001$, resp.; Figure 2). In addition, a statistically significant positive correlation was identified between serum α2-anti-plasmin and HbA1c levels ($r = 0.268$, $P = 0.031$).

4. Discussion

In previous studies, it was shown that almost 90% of patients with insulin-dependent diabetes mellitus had retinopathy 10 years after disease onset. After a disease duration of 20 years, this percentage becomes 99%, of which 53% is proliferative and sight threatening [11]. In our study, the prevalence of proliferative retinopathy was positively correlated with disease duration.

The classical factors that are predictive for occurrence of diabetic retinopathy are disease duration, the age at onset and examination, control of diabetes, presence and regulation of hypertension, or proteinuria, and serum creatinine levels. Chronic hyperglycemia during diabetes is the major contributing and well known factor to the occurrence of microvascular complications [12]. Previous studies have demonstrated that the alterations in prothrombotic/inflammatory markers which are related with diabetes and cardiovascular risk develop at an early stage of disease development, and can be partly improved by strict glycemic control [13]. However, the pathogenesis of diabetic retinopathy (DR) is not completely understood, and is suggested to involve inflammation and endothelial dysfunction [14].

The vascular endothelium that constitutes the primary defense against thrombosis is abnormal in diabetes, which might result in the characteristic increased activation of platelets and clotting factors [15]. The pathogenesis of type I DM differs from type II, and there are also differences in the prothrombotic processes. In patients with type I diabetes, the activation of coagulation is the prominent pathway [7, 8], whereas impaired fibrinolysis dominates in type II diabetes [16].

An association between hyperfibrinogenemia and the occurrence of diabetic macroangiopathy was reported [17, 18]. In a study by Asakawa et al., increased fibrinogen levels were reported as a risk factor for retinopathy in type II diabetes patients [19]. Fibrinogen levels were also found to be increased significantly in patients with background retinopathy.

However, the relationship between fibrinogen levels and microangiopathy remains unclear and there are conflicting reports in the literature. For instance, Hirano et al. did not detect differences in fibrinogen levels between diabetic patients with normo-, micro-, and macroalbuminuria [20]. Unlike previous studies, our data purely included patients with type I diabetes, and we did not detect any differences in serum fibrinogen levels between groups.

Elevated serum concentrations of PAI-1 are frequently observed in patients with diabetes, particularly those with type 2 diabetes [21]. Metabolic derangements such as hyperglycemia and hypertriglyceridemia occur due to insulin deficiency and may contribute to the elevated concentrations of PAI-1 observed *in vivo* [22, 23]. However, reports on plasma levels of PAI-1 in type 1 diabetes are contradictory. In the present study, serum PAI-1 levels were slightly higher in the diabetic groups than control, but the differences were not statistically significant ($P = 0.209$). In a previous study of 35 patients, the basal PAI-1 levels of patients with type 1 diabetes were higher than the control group. In contrast, Kırmızıbekmez et al. reported that there was no correlation between serum PAI-1 levels and disease duration or the presence microvascular complications in eighty-four Turkish type 1 diabetic children with a disease duration of more than 5 years [24] which is consistent with our data and a previous study [6].

We observed that serum plasminogen levels were similar between the diabetic subgroups and control. In contrast, levels of α2-anti-plasmin were increased significantly in the diabetic groups, compared with control, and were correlated with HbA1c measurement. Although serum α-anti-plasmin level was higher in Group III compared to other diabetic subgroups (Group I and II), the difference was not statistically significant. To our knowledge, there are only a small number of studies evaluating α2-anti-plasmin levels in Type 1 diabetes, although findings are consistent with our observations. In a recent study, the plasma plasmin-α2-anti-plasmin complex (PAP) was associated with diabetic retinopathy and sight threatening diabetic retinopathy in Type 2 diabetics [10], and correlation was still significant after adjustments of the groups for age, gender, race, the study center, SBP, the use of diabetes medications, disease duration, HbA1c, and waist-to-hip ratio were made [25, 26]. A positive correlation between α2-anti-plasmin with HbA1c suggests

that fibrinolytic markers may improve with disease regulation and better glycemic control.

5. Conclusions

Diabetes is a state associated with increased risk for thrombosis and it does not only contribute to major vessel diseases but also to microvascular complications. However, additional studies on larger patient populations are necessary to reveal the fibrinolytic and thrombotic status of patients with Type 1 diabetes. Confirmation of these data would allow a better understanding of the pathogenesis of DR, which may lead to the development of therapies that prevent its onset and/or progression.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publishing of this paper.

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Clinical Study

Relation of Asymmetric Dimethylarginine Levels to Macrovascular Disease and Inflammation Markers in Type 2 Diabetic Patients

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Aim. We aimed to determine the relation of asymmetric dimethyl arginine (ADMA) levels to atherosclerotic vascular disease and inflammation markers in type 2 diabetes. *Methods*. We recruited 50 type 2 diabetic patients with atherosclerosis, 50 type 2 diabetic patients without atherosclerosis, and 31 healthy control patients into our study. We obtained fasting serum and plasma samples and measured HbA1c, fasting blood glucose, C-peptide, creatinine, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, hsCRP, fibrinogen, erythrocyte sedimentation rate, total homocysteine, and ADMA levels. In addition, all of the patients were evaluated for carotid artery intima media thickness by ultrasound. We evaluated ADMA levels in healthy controls, diabetic patients with macrovascular complications, and diabetic patients without macrovascular complications and evaluated the relationship between ADMA levels and total homocysteine, inflammation markers, and macrovascular disease. *Results*. Mean ADMA values in non-MVD and control groups were significantly lower than in MVD group (0.39 ± 0.16, 0.32 ± 0.13, 0.52 ± 0.23, $P < 0.05$, resp.). These three variables (carotid intima-media thickness, inflammatory markers, and ADMA levels) were significantly higher in diabetes group than control ($P < 0.05$). *Conclusion*. There is a relationship between ADMA and macrovascular disease in type 2 diabetes, but further studies are needed to understand whether increased ADMA levels are a cause of macrovascular disease or a result of macrovascular disease.

1. Introduction

Type 2 diabetes mellitus (DM) is one of the underlying causes of patient morbidity and mortality and is an important risk factor for coronary artery disease development. Compared to healthy populations, type 2 diabetic patients have a 2-3 times higher risk of macrovascular disease [1]. Coronary artery disease resulting from diabetes is responsible for 75% of diabetes related deaths [2].

Mechanical or functional damage of the endothelium results in diminished blood vessel function and can initiate the development of atherosclerosis. Studies have shown that the presence of endothelial dysfunction is an important prognostic indicator for future cardiovascular events [3, 4]. Endothelial dysfunction, on the other hand, can also result from decreases in free radicals, which are derived from oxygen and/or endothelial nitric oxide synthase (eNOS) activity and/or expression.

Asymmetric dimethylarginine (ADMA), an NOS inhibitor, is considered an indicator of endothelial dysfunction due to increased expression of ADMA in renal failure, coronary artery disease, apoplexy, hypertension, and diabetes mellitus [5, 6].

The idea that ADMA levels may not only be an indicator of endothelial dysfunction but may also play a role in increasing endothelial dysfunction demonstrates the need for further research. Atherosclerosis is considered an inflammatory process and increases in inflammation markers reflect the atherosclerotic process. These studies suggest that more information could be gathered and new treatment modalities could be developed in the prevention of endothelial dysfunction and atherosclerosis.

In this study, we examined the relationship between the ADMA levels in type 2 DM patients and indicators of macrovascular disease and inflammation.

2. Materials and Methods

Out of the 131 patients recruited into the study, 100 were diagnosed with type 2 DM, half of which also had atherosclerotic vascular disease. The remaining 31 patients were healthy. The research was reviewed and approved by the Eskişehir Osmangazi University Ethics Committee.

Patients with a history of myocardial infarction, coronary artery bypass operation, peripheral artery disease, and an apoplexy incident, ECG findings that hinted angina pectoris or ischemia or angiographically proven coronary artery disease were included in the macrovascular disease (MVD) group. Type 2 diabetic patients who did not have any known myocardial infarction, coronary artery bypass operation, peripheral artery disease, apoplexy, or angiographically proven coronary artery disease were included in non-MVD group. Patients with no evidence of pathology were included in the control group after receiving a verbal record of their mental history and a physical examination. Age, sex, BP levels, duration of diabetes, types of treatment, additional diseases of the patients,and the medicine prescribed were all noted.

Preprandial serum and plasma samples obtained from patients were tested for HgA1c, glucose, C-Peptide, total cholesterol, LDL, creatinine, hsCRP, fibrinogen, and erythrocyte sedimentation rate. Patients with hypothyroidism, vitamin B12 or folic acid deficiency after thyroid function tests were excluded from the study. The patients with creatinine levels above 1.8 mg/dL were not included in the study to eliminate any possible effects of their uremia. Patients recently diagnosed with diseases that could affect inflammation parameters, such as diabetic foot infection, were also excluded from the study.

Preprandial serum samples were kept at −70°C until total homocysteine (tHcy) (using Immulite 2000 Homocysteine kit, Siemens Medical Solutions Diagnostic, USA, and Competitive Immunoassay method) and ADMA (using ADMA direct ELISA Kit, Immunodiagnostic AG, Bensheim, Germany) were measured. Carotid artery intima media thickness was ultrasonographically evaluated.

The correlation between ADMA levels, tHcy, inflammatory markers and MVD was determined in the healthy control group, in diabetic patients with MVD and in diabetic patients non-MVD.

Statistical Analysis. Descriptive statistics of the data were summarized as mean ± standard deviation. The Shapiro-Wilk test was used to determine whether variables were normally distributed. Parametric tests were conducted on normally distributed variables and nonparametric tests on variables with nonnormal distributions. Student's t-test and Mann-Whitney U test were used to compare two independent groups' means and medians, respectively. One-way ANOVA was used for comparison of three groups' means and Kruskal-Wallis test was used for differences among the groups' medians. The relationships between two categorical variables were evaluated by using chi-square test. Correlation analysis was used for the relation between numerical variables in each group. The linear relationships between ADMA and risk factors were evaluated by using multiple linear regression models. $P < 0.005$ was considered statistically significant. SPSS for Windows 15.0 was used for statistical analyses.

3. Results

Fifty patients with MVD and 50 patients with non-MVD, comprising the 100 type 2 diabetic patients in addition to 31 healthy control patients, were recruited for the study. The relationships of the variables age, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), duration of diabetes (D. of Diabetes), HbA1c, CrCl, albuminuria, PBG, c-peptide, creatinine, triglyceride, total cholesterol, HDL, LDL, sedimentation, hsCRP, fibrinogen, tHcy, vitamin B12, folic acid, right carotid intima media thickness (Car.Int-R), left carotid intima media thickness (Car.Int-L), ADMA to one another in MVD, non-MVD, and the control group, respectively, were evaluated by one-way ANOVA and Kruskal-Wallis tests (Table 1). The relationships of the common variables to one another in diabetic and healthy patients were also evaluated by t-test and Mann-Whitney U test (Table 2).

Sedimentation, CRP, and fibrinogen levels were measured as markers of inflammation and groups with respect to these three markers were compared. Mean of these 3 markers in MVD group compared to the control group were higher, but the differences between MVD and non-MVD, non-MVD, and control groups were not found significantly.

Mean ADMA value in non-MVD and control groups were significantly lower than in MVD group, respectively ($P < 0.05$ for each). According to these results, ADMA may be associated with macrovascular complications. In terms of levels of ADMA, there was no significant difference between the non-MVD and control groups.

The mean value of Car.Int-R variable in MVD group was found to be higher than control group ($P < 0.05$). In addition the mean of MVD and non-MVD groups were found to be higher than control group with regard to Car.Int-L.

The data were divided into a control group and patients group with all diabetes, and these two groups were compared in terms of common measurements. The results obtained are given in Table 2. There was no significant difference between the two groups in terms of HDL but triglyceride levels were higher in diabetic group than the control group. Cholesterol levels and LDL cholesterol levels in the control group were significantly higher than those of diabetes (P values < 0.05 and < 0.01, resp.). This result is connected to diet factor and statin use of the diabetic patients.

The mean Car.Int-R and Car.Int-L values, which were recognized as one of the important markers of atherosclerosis, of patients with diabetes are significantly higher than the control group ($P < 0.01$, for each). This result showed once again that diabetes is a major risk factor for atherosclerosis.

Sedimentation, hsCRP and fibrinogen levels were measured as markers of inflammation. Two groups compared

Table 1: Comparison of the common parameters in MVD, non-MVD, and the control group.

	MVD (a)	non-MVD (b)	Control (c)	
Age (years)	61.86 ± 7.96	56.8 ± 8.94	55.06 ± 7.46	(a-c) $P < 0.001$, (a-b) $P < 0.01$
BMI (kg/m^2)	29.59 ± 4.76	30.44 ± 6.22	27.83 ± 2.68	$P > 0.05$
SBP (mm/Hg)	126.20 ± 13.07	125.40 ± 15.31	121.77 ± 9.53	$P > 0.05$
DBP (mm/Hg)	80.80 ± 8.16	77.90 ± 12.86	80.80 ± 8.76	$P > 0.05$
D. of diabetes (Years)	12.98 ± 7.24	9.08 ± 5.75		$P < 0.01$
HbA1c (%)	9.41 ± 2.63	9.88 ± 2.93		$P > 0.05$
CCr (mL/min)	84.25 ± 40.55	95.27 ± 42.38		$P > 0.05$
Albuminuria (mg/day)	305.20 ± 946.28	110.70 ± 238.78		$P > 0.05$
C-Peptide (ng/mL)	2.21 ± 1.77	2.17 ± 1.23		$P > 0.05$
PBG (mg/dL)	171.56 ± 76.20	202.72 ± 99.52	86.87 ± 8.65	(b-c) $P < 0.05$, (a-c) $P < 0.05$
Creatinine (mg/dL)	1.01 ± 0.25	0.85 ± 0.18	0.82 ± 0.15	(a-b) $P < 0.05$, (a-c) $P < 0.05$
Triglyceride (mg/dL)	188.68 ± 109.11	185.44 ± 101.35	141.19 ± 78.74	(a-c) $P < 0.05$, (b-c) $P < 0.05$
HDL (mg/dL)	50.80 ± 13.95	49.74 ± 10.31	54.20 ± 13.18	$P > 0.05$
T. cholesterol (mg/dL)	181.26 ± 46.90	189.16 ± 43.81	203.38 ± 33.94	$P > 0.05$
LDL (mg/dL)	113.40 ± 36.89	121.50 ± 34.24	137.58 ± 32.56	(c-a) $P < 0.01$, (c-b) $P < 0.05$
Sedimentation (mm/hr)	25.38 ± 21.88	18.60 ± 15.35	12.51 ± 8.31	(a-c) $P < 0.05$
CRP (mg/L)	7.71 ± 9.21	6.57 ± 11.42	2.36 ± 1.85	(a-c) $P < 0.05$
Fibrinogen (mg/dL)	386.66 ± 91.74	370.32 ± 68.31	344.51 ± 55.69	(a-c) $P < 0.05$
Homocysteine (umol/L)	11.78 ± 3.85	11.38 ± 3.86	10.58 ± 3.59	$P > 0.05$
B12 (pg/mL)	537.84 ± 381.73	449.14 ± 309.35	421.03 ± 274.66	$P > 0.05$
Folic acid (ng/mL)	9.27 ± 3.57	9.51 ± 3.58	9.93 ± 8.14	$P > 0.05$
Car.Int-R (mm)	0.96 ± 0.23	0.89 ± 0.24	0.78 ± 0.19	(a-c) $P < 0.05$
Car.Int-L (mm)	0.93 ± 0.18	0.93 ± 0.28	0.79 ± 0.19	(a-c) $P < 0.05$, (b-c) $P < 0.05$
ADMA (umol/L)	0.52 ± 0.23	0.39 ± 0.16	0.32 ± 0.13	(a-b) $P < 0.05$, (a-c) $P < 0.05$

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; D. of diabetes: duration of diabetes.

Table 2: Comparison of the common parameters in all diabetic patients and the control group.

	DM	Control	P values Significance
Age (years)	59.41 ± 8.81	55.00 ± 7.34	<0.05
Creatinine (mg/dL)	0.93 ± 0.23	0.82 ± 0.16	<0.05
Triglyceride (mg/dL)	187.65 ± 105.15	140.78 ± 77.49	<0.01
T. cholesterol (mg/dL)	185.78 ± 45.18	201.03 ± 35.95	<0.05
HDL (mg/dL)	50.36 ± 12.24	53.79 ± 13.17	>0.05
LDL (mg/dL)	117.91 ± 35.51	135.50 ± 34.13	<0.01
Sedimentation (mm/hr)	21.96 ± 19.21	12.87 ± 8.42	<0.01
hsCRP (mg/L)	7.21 ± 10.37	2.29 ± 1.86	<0.01
Fibrinogen (mg/dL)	377.86 ± 81.06	347.50 ± 57.32	<0.01
Homocysteine (umol/L)	11.58 ± 3.86	10.61 ± 3.53	>0.05
Car.Int-R. (mm)	0.92 ± 0.22	0.80 ± 0.22	<0.01
Car.Int-L (mm)	0.93 ± 0.23	0.80 ± 0.20	<0.01
ADMA (umol/L)	0.45 ± 0.21	0.32 ± 0.13	<0.001

with each other about these three markers, means of these 3 markers in diabetes group were found higher than control group ($P < 0.01$ for each). In addition we showed that the mean ADMA value of diabetic group were significantly higher than the control group's ($P < 0.001$).

As a result, because "These three variables (carotid intima-media thickness, inflammatory markers, ADMA levels) were significantly higher in diabetes group than control ($P < 0.05$)", while in that "Because these three variables (carotid intima-media thickness, inflammatory markers, ADMA levels) were significantly higher than diabetes group than control ($P < 0.05$)".

In the first two tables, inflammatory markers, creatinine, and ADMA levels which tended to increase with macrovascular disease and with diabetes are shown.

Later in the study, the correlations between ADMA levels and creatinine, inflammatory markers (sedimentation, hsCRP, and fibrinogen), and tHcy levels in all groups were evaluated. The results obtained are presented in Table 3.

ADMA and creatinine levels were positively correlated ($P < 0.05$) (Table 3). But there were no significant relationships between ADMA and sedimentation, hsCRP, fibrinogen, and tHcy.

In the MVD group, the relationships between ADMA and creatinine levels, sedimentation, hsCRP, fibrinogen, and tHcy were not found significant (Table 4).

In addition ADMA was evaluated by multiple regression analysis in which ADMA was taken as a dependent variable and T. cholesterol, PBG, C peptide, duration of diabetes, age, sedimentation, Car.Int-R, Car.Int-L, tHcy, hsCRP, BMI, HDL,

Table 3: Correlations between ADMA and creatinine, sedimentation, hsCRP, fibrinogen, and homocysteine in all groups.

	ADMA	
Creatinine (mg/dL)	$r = 0.173$	$P < 0.05$
Sedimentation (mm/hr)	$r = 0.059$	$P > 0.05$
hsCRP (mg/L)	$r = 0.098$	$P > 0.05$
Fibrinogen (mg/dL)	$r = 0.057$	$P > 0.05$
Homocysteine (umol/L)	$r = 0.069$	$P > 0.05$

Table 4: Correlations between ADMA and creatinine, sedimentation, hsCRP, fibrinogen, and homocysteine in MVD group.

	ADMA	
Creatinine (mg/dL)	$r = 0.024$	$P > 0.05$
Sedimentation (mm/hr)	$r = 0.00$	$P > 0.05$
hsCRP (mg/L)	$r = 0.217$	$P > 0.05$
Fibrinogen (mg/dL)	$r = 0.269$	$P > 0.05$
Homocysteine (umol/L)	$r = 0.189$	$P > 0.05$

Table 5: Multiple regression analysis results in MVD group.

Independent predictors	β	P
Albuminuria (mg/day)	−0.322	0.21
Triglyceride (mg/dL)	0.254	0.63
Homocysteine (umol/L)	0.403	**0.002**
Car.Int-R (mm)	−0.221	0.81

HgA1c, CrCl, creatinine, albuminuria, fibrinogen, triglyceride, and LDL were taken as independent variables in MVD group. As a result, we showed that tHcy levels had significant effects on ADMA in Table 5. The relation between tHcy levels and ADMA was positive.

4. Discussion

Type 2 diabetes mellitus is one of the main causes of morbidity and mortality in the world, and its prevalence has increased dramatically in the past ten years [7]. Atherosclerosis is one of the underlying causes of morbidity and mortality in patients with type 2 DM, and type 2 diabetic patients are 2-3 times more likely to develop macrovascular disease [1].

Late complications from microangiopathy and macroangiopathy are burdens on both patient health and the economy. In treating and preventing type 2 DM the principal aim should be not merely to decrease the incidence of metabolic disease but also to minimize microvascular and macrovascular complications [8].

Patients at risk for atherosclerosis often have endothelial dysfunction. The relationship between oxidative stress, which increased by the NOS inhibitor ADMA, and endothelial dysfunction has been studied in many experimental animal models and diseases. Many studies have shown that increased ADMA in endothelial dysfunction is related to an increase in the generation of reactive oxygen species in plasma [8–10]. It has also been shown that endothelial dysfunction in coronary and peripheral vascular disease is connected with increased plasma ADMA concentration [11].

Studies have shown that ADMA levels increase in patients with cardiovascular risk [12, 13]. Macrovascular disease is 2-3 times more common in type 2 diabetics compared to normal population [1]. Krzyzanowska and colleagues showed that ADMA is related to clinical macrovascular atherosclerotic disease diagnosis in type 2 diabetics [14]. Research has also shown that increased tHcy concentration in type 2 diabetics is associated with increased cardiovascular disease [15, 16]. In our study, the ADMA level was found to be higher in diabetic patients with macrovascular complications compared to diabetic patients without complications. In addition, when all diabetic patients were compared to the control group, the ADMA level was higher in the diabetic group. We hypothesized that ADMA might be related to macrovascular atherosclerotic disease in type 2 diabetics. Moreover Krzyzanowska et al. concluded that ADMA is associated with tHcy, albuminuria, creatinine, and GFR and that tHcy correlates with age, ADMA, creatinine, GFR, LDL, and DTA [14]. In our study, we found a positive correlation between ADMA and age in diabetic patients with macrovascular complications and also found a correlation between ADMA and creatinine in all patients. No statistically significant relation between ADMA and tHcy was found in our study. Additionally, using ADMA as a dependent variable in multiple regression analysis, the levels of fundamental determinants of ADMA were evaluated in diabetic patients with macrovascular complications. It was found that ADMA's most fundamental determinant is tHcy. The data showed that ADMA is increased during atherosclerosis and DM, that ADMA is involved in the development of atherosclerosis, and that the fundamental determinant of ADMA is tHcy. Thus, ADMA may be used to predict the likelihood of developing macrovascular disease in diabetic patients.

Correlations between the levels of ADMA [17] and tHcy [18] and increased carotid intimamedia thickness during atherosclerosis development have been shown. In our study, ADMA levels were found to be much higher in diabetic patients with macrovascular complications than in the control group. When all diabetics were compared to the control group, the ADMA level in the diabetic group was found to be significantly higher. Also, carotid intimamedia thickness values in diabetic patients with macrovascular complication were higher than in the control group. When all diabetics were compared to the control group, carotid intimamedia thickness values in all diabetics were found to be higher than in the control group. However, using correlation analysis, no significant relation between ADMA and carotid intimamedia thickness was found. In addition, there was no significant difference in tHcy levels between groups. However, when all diabetics were considered there was a positive correlation between tHcy and carotid intimamedia thickness. The fact that ADMA levels in diabetic patients with macrovascular complications were higher made us question whether ADMA correlates with atherosclerotic development in the control group. In addition, since ADMA in diabetic patients was higher compared to the control group, we hypothesized that ADMA might have a role in atherosclerotic development in

patients with DM, which is one of the main risk factors of atherosclerosis.

Various studies have shown that the inflammatory factor CRP level in type 2 diabetics is indicative of cardiovascular risk [19–21]. Moreover, Krzyzanowska and colleagues showed that ADMA is a new indicator of cardiovascular risk in type 2 diabetics and that it, independently of other known risk factors, increases the predictive value of CRP for cardiovascular disease [22]. Yet another study found that ADMA correlates with macrovascular disease and subclinical inflammation in type 2 diabetics [23]. In our study, inflammatory markers hsCRP, sedimentation, and fibrinogen were measured. All three inflammatory factors were significantly higher in diabetic patients with macrovascular complication than in the control group. Also, when all the diabetics were compared with the control group, the levels of the inflammatory markers were dramatically higher in the diabetic patients. However there was no significant correlation between ADMA and the inflammatory indicators. Corresponding to the existing evidence in diabetics, ADMA might be an indicator of macrovascular disease independent from inflammatory markers.

Consequentially, it is accepted that ADMA levels, a NOS inhibitor, can be used to diagnose macrovascular disease and cardiovascular mortality risk in type 2 diabetics. In our study, we concluded that ADMA's significance in the diagnosis of macrovascular disease risk is independent from other inflammatory markers. Thus, ADMA should be considered independently of the inflammatory markers in the prediction of macrovascular disease. Nevertheless it is not clear whether ADMA is a result of macrovascular disease development or a causative factor in macrovascular disease development. For this reason additional studies are needed to elucidate the effects of treatments targeted at decreasing ADMA levels and thus decreasing macrovascular disease development.

Results.

(1) ADMA levels in diabetic patients with macrovascular complications were significantly higher than in patients without macrovascular complications and the control group ($P < 0.05$). Thus, ADMA might be related to macrovascular complication development.

(2) When all the diabetics were compared to the control group, ADMA levels in the diabetics were significantly increased ($P < 0.001$). It was shown that ADMA tends to increase in DM, which is a main cause of atherosclerosis.

(3) Sedimentation, hsCRP, and fibrinogen levels, which are inflammatory markers, were significantly higher in diabetic patients with macrovascular complications than in the control group ($P < 0.05$).

(4) When all the diabetics were compared to the control group, the inflammatory markers sedimentation, hsCRP, and fibrinogen levels were significantly higher compared to the control group ($P < 0.01$). It was shown that inflammatory markers might be indicative of macrovascular complications and also that these markers increase in DM.

(5) No significant difference in tHcy levels was found between the groups.

(6) No significant correlation was found between ADMA and inflammation markers or homocysteine. This suggests that ADMA might be causing macrovascular complications independently of homocysteine and the inflammatory markers.

(7) The levels of fundamental determinants of ADMA were evaluated in diabetic patients with macrovascular complications using multiple regression analysis in which ADMA was the dependent variable. We found that ADMA's most fundamental determinant is homocysteine.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Urinary Methylmalonic Acid as an Indicator of Early Vitamin B12 Deficiency and Its Role in Polyneuropathy in Type 2 Diabetes

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The rising incidence of diabetes and its negative impact on quality of life highlights the urgent need to develop biomarkers of early nerve damage. Measurement of total vitamin B12 has some limitations. We want to determine the levels of urinary methylmalonic acid and its relationships with serum vitamin B12 and polyneuropathy. The 176 Chinese patients with Type 2 diabetes mellitus were divided into 3 groups according to the levels of vitamin B12. A gas chromatography mass spectrometric technique was used to determine blood methylmalonic acid and urinary methylmalonic acid. The diagnosis of distal diabetic polyneuropathy was based on the determination of bilateral limb sensory and motor nerve conduction velocity and amplitude with electromyogram. Multiple regression analysis revealed that urinary methylmalonic acid/creatinine, blood methylmalonic acid, and so forth were variables that influenced diabetic polyneuropathy significantly. Nerve sensory conduction velocity and nerve amplitude in the group of urinary methylmalonic acid/creatinine >3.5 mmol/mol decreased significantly. Superficial peroneal nerve sensory and motor conduction velocity and ulnar nerve compound motor active potential amplitude were inversely correlated with urinary methylmalonic acid/creatinine. Urinary methylmalonic acid correlates with serum vitamin B12 levels in person with diabetes and is a sensitive marker of early polyneuropathy.

1. Introduction

Diabetes mellitus (DM) is caused by genetic and environmental interactions, along with changing lifestyles and an aging population, increasing incidence of diabetes. Diabetic neuropathy is one of the major complications of diabetes with both Type 1 and Type 2. Up to 50% of all diabetes have polyneuropathy which is a major cause of morbidity and associated with increased mortality, and up to 26% of diabetics develop painful diabetic neuropathy with debilitating effects on quality of life [1–3].

The rising incidence of diabetes and its negative impact on quality of life highlights the urgent need to develop biomarkers of early nerve damage. The gold-standard method to evaluate morphological change in small nerve fibres was the skin biopsy [4]; however, this technique is limited by cost and invasiveness, provides no information about the function of nerve fibres, and cannot be employed as a generalized screening test in all patients.

Vitamin B12 (Vit B12) is a cofactor for methylmalonylCoA mutase, which converts methylmalonyl CoA to succinyl CoA. Measurement of total Vit B12 suffers from some limitations; in particular, most of the cobalamin measured is that bound to haptocorrin (HC) [5, 6]. Methylmalonic acid (MMA) may contribute to neuronal injury in many human conditions in which it accumulates, including methylmalonyl-CoA mutase and Vit B12 deficiency [7, 8]. The impaired activity of the enzyme leads to an accumulation of MMA and an elevated

plasma concentration [9]. MMA is biochemically more stable in urine than in serum and has a 40-fold greater concentration in urine [10]. Urinary methylmalonic acid (uMMA) concentration offers a potentially useful functional marker of Vit B12 status. Moreover, the measurement of uMMA would be a less invasive method for the purpose of screening or epidemiologic studies. In addition, uMMA is excreted very efficiently by the kidneys, which concentrates the metabolite in the urine and makes it a sensitive indicator of tissue depletion [10].

Based on the above considerations, we believed that uMMA was an intermediate metabolite during the course of neuropathy, and there was an important relationship between uMMA and the formation of neuropathy. The aim of the present study was to assess uMMA levels in patients presenting with and without diabetic polyneuropathy (DPN) and to determine the role of uMMA in DPN.

2. Subjects, Materials, and Methods

2.1. Subject Selection. The study population consisted of 176 Chinese Han patients (male 97 and female 79) with Type 2 diabetes mellitus aged 38–70 y recruited upon admission to the Second Hospital of Shandong University between June in 2009 and June in 2011 who were eligible for the study.

Exclusion criteria were as follows: history of serious heart and lung disease, diabetic kidney disease or other kidney diseases, pernicious anemia, intestinal surgery, and gastrointestinal disease, excluding antibiotics, colchicine, aminosalicylic acid, H2 receptor antagonists and proton pump inhibitors and other effects of gastrointestinal motility drugs, and the patients with the Vit B12 or methylcobalamin treatment. Long-term vegetarians (more than six months) and those refused concurrent electrophysiological or laboratory testing were excluded too.

According to the level of Vit B12, the patients were divided into 3 groups: group A (Vit B12 < 180 ng/L, $n = 58$), group B (Vit B12 180–400 ng/L, $n = 68$), and group C (Vit B12 > 400 ng/L, $n = 50$).

2.2. Detection of Indicators. The fasting blood samples (22 mL/person) via venipuncture into EDTA-coated tubes (5 mL) for a full blood were counted to check glycosylated hemoglobin A1c (HbA1c), triglycerides (TG), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), serum creatinine (sCr), Vit B12, folic acid, ferritin, homocysteine (Hcy), hemoglobin (HGB), and mean corpuscular volume (MCV). Holotranscobalamin (holoTC) level was determined by microparticle enzyme immunoassay on Axsym analyzer (both from Abbott, USA). Fasting morning urine samples were for use. Serum samples were stored at −80°C. Approximately 10 mL fasting urine samples were stored at −80°C untreated.

The application of stable isotope dilution method for rapid extraction of the solid phase sample by gas chromatography mass spectrometry was used for the determination of blood methylmalonic acid (bMMA) and uMMA. Urinary MMA was expressed relative to urinary creatinine as the uMMA/creatinine.

Diabetic polyneuropathy: the diagnosis of bilateral limb sensory and motor nerve conduction velocity (SNCV/MNCV) and amplitude was performed, according to standard techniques protocols, by means of Keypoint, Dantec equipment, for all individuals. Tests were carried out in a warm room to minimize the effects of temperature on nerve conduction velocity (CV), and the limbs were warmed throughout with an infrared lamp to maintain a skin temperature over 35°C. Suprathreshold stimulation was used, the frequency was 1 Hz (feeling) and 1 Hz (movement), respectively, and course was both for 0.2 ms. Nerve conduction and electromyographic studies were performed in four limbs, which included 2 nerves of upper limbs and 2 nerves of lower limbs, by means of standard techniques. We measured the amplitude and velocity of motor nerve conduction and sensor nerve conduction in 4 nerves: superficial peroneal, sural, median, and ulnar.

2.3. Statistical Analysis. All experimental data were analysed by SPSS software 16.0 (SPSS, Chicago, IL, USA). Measurement data were presented as mean ± standard deviation (mean ± SD). Single factor and multifactor ANOVA analysis were for the mean difference among the groups. Correlation was analysed using Pearson correlation. The count data were analyzed by χ^2 test, and logistic regression analysis was used for an independent risk factor of diabetic polyneuropathy. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Analysis of Clinical Characteristics (See Table 1). Patients in the two groups with normal Vit B12 had decreased serum concentrations of HDL, ferritin, folic acid, holoTC, and HGB and increased uMMA/creatinine and bMMA compared to those lack Vit B12. In addition, DM Type 2 patients with Vit B12 over 400 ng/L had significantly increased ferritin, holoTC, and HGB and decreased uMMA/creatinine compared to those with Vit B12 180–400 ng/L. There was no significant difference in LDL, MCV, duration, sCr, TG, BMI, and HbA1c among the three groups. Polyneuropathy was present in 24.7% of our diabetic patients using electromyogram.

3.2. Multiple Regression Analysis. Multiple regression analysis revealed that uMMA/creatinine ($P = 0.001$), bMMA ($P = 0.02$), diabetes duration ($P = 0.045$), and holoTC ($P = 0.003$) were variables that influenced DPN significantly and independently while the serum concentration of Vit B12 did not have an independent significant influence on DPN (Table 2).

3.3. Prevalence of Electrophysiological Markers of Neuropathy Severity according to Urinary Methylmalonic Acid/Creatinine. To value the relationship between uMMA/creatinine and DPN, we separated data according to whether uMMA/creatinine was normal or elevated. It was observed that nerve sensory conduction velocity and nerve amplitude in the group of uMMA/creatinine >3.5 mmol/mol decreased significantly. All individual nerve conduction parameters

Table 1: Comparison of the clinical data among the three groups.

Parameters	Group A	Group B	Group C
Age (year)	58.24 ± 10.53	55.81 ± 10.83	56.98 ± 10.72
Male/female	30/28	36/32	28/22
Duration (year)	6.28 ± 6.6	4.67 ± 5.88	4.13 ± 4.77
Vitamin B12 (ng/L)	127.53 ± 60.71	303.62 ± 109.98	662.86 ± 260.92
BMI (Kg/m^2)	26.06 ± 3.61	26.10 ± 3.31	25.93 ± 4.09
Hemoglobin A1c (%)	9.12 ± 2.33	9.42 ± 2.13	8.38 ± 1.97
Triglycerides (mmol/L)	1.79 ± 0.86	2.12 ± 1.81	1.81 ± 0.88
High-density lipoprotein cholesterol (mmol/L)	1.20 ± 0.24	1.31 ± 0.28*	1.39 ± 0.31*
Low-density lipoprotein cholesterol (mmol/L)	2.72 ± 0.80	2.84 ± 0.63	2.01 ± 1.23
Serum creatinine (umol/L)	60.00 ± 4.32	65.89 ± 4.71	61.08 ± 4.46
Hemoglobin (g/L)	130.10 ± 18.54	136.65 ± 14.58*	146.71 ± 10.01*#
Mean corpuscular volume (fL)	85.32 ± 5.60	86.66 ± 8.36	87.38 ± 9.98
Ferritin (ug/L)	166.44 ± 92.51	225.18 ± 103.06*	259.33 ± 119.33*#
Folic acid (ng/L)	6.17 ± 2.06	6.50 ± 2.18*	6.58 ± 2.32*
Holotranscobalamin (pmol/L)	54.82 ± 30.47	70.10 ± 26.00[a]	81.32 ± 32.91[ab]
Urinary methylmalonic acid/creatinine	8.04 ± 2.19	4.15 ± 1.06[a]	2.75 ± 0.71[ab]
Blood methylmalonic acid (nmol/L)	400.09 ± 80.52	370.57 ± 110.9[a]	350.72 ± 120.02[a]
Metformin drug usage (%)	80	76	79
Polyneuropathy (%)	35	21	18

Values are means ± SD. *$P < 0.05$ compared to group A; #$P < 0.05$ compared to group B; [a]$P < 0.01$ compared to group A; [b]$P < 0.01$ compared to group B.

Table 2: Logistic analysis of influencing factors of diabetic polyneuropathy.

Parameters	OR value (95% CI)	P value
Age (year)	0.73 (0.84–1.08)	0.59
Duration (year)	1.543 (1.302–1.829)	0.045
Low-density lipoprotein cholesterol (mmol/L)	0.76 (0.63–0.89)	0.587
High-density lipoprotein cholesterol (mmol/L)	1.03 (0.81–1.42)	0.407
Triglycerides (mmol/L)	0.27 (0.11–0.36)	0.816
Serum creatinine (umol/L)	1.15 (0.79–1.54)	0.313
HbA1c (%)	1.19 (0.85–1.43)	0.254
Mean corpuscular volume (fL)	0.18 (0.11–0.25)	0.884
Hemoglobin (g/L)	0.99 (0.70–1.27)	0.458
Ferritin (ug/L)	1.08 (0.93–1.21)	0.340
Folic acid (ng/L)	0.92 (0.86–0.96)	0.532
Urinary methylmalonic acid/creatinine (mmol/mol)	4.07 (3.15–5.46)	0.001
Blood methylmalonic acid (pg/mL)	2.152 (1.799–2.42)	0.02
Vitamin B12	0.88 (0.794–0.997)	0.547
Holotranscobalamin (pmol/L)	3.89 (2.77–4.56)	0.003

except median nerve sensory and motor conduction velocity and ulnar nerve motor conduction velocity were statistically significant between the two groups (see Table 3).

3.4. The Correlation Analysis between Urinary Methylmalonic Acid/Creatinine and Electrophysiological Markers of Neuropathy Severity. Univariate analysis was carried out with the uMMA/creatinine as the outcome variable. Some significant correlations were revealed between the uMMA/creatinine and other electrophysiological markers of neuropathy severity (Table 4). Superficial peroneal nerve sensory and conduction velocity ($r = -0.496$, $P = 0.0005$; $r = -0.327$, $P = 0.003$, resp.) and ulnar nerve sensory active nerve potential amplitude ($r = -0.315$, $P = 0.005$) were inversely correlated with the uMMA/creatinine. Sural nerve compound motor active potential amplitude ($r = -0.196$, $P = 0.02$), sural nerve motor conduction velocity ($r = -0.205$, $P = 0.01$), superficial peroneal nerve compound motor active potential

Table 3: Prevalence of electrophysiological markers of neuropathy severity according to urinary methylmalonic acid/creatinine.

Electrophysiological markers of neuropathy severity	uMMAr > 3.5 mmol/mol	uMMAr < 3.5 mmol/mol	P
Sural nerve SNAP amplitude (μV)	1.45 ± 0.41	2.31 ± 1.02	0.03
Sural nerve sensory conduction velocity (m/s)	45.35 ± 6.11	55.98 ± 9.25	0.04
Sural nerve CMAP amplitude (μV)	1.12 ± 0.29	2.09 ± 0.91	0.001
Sural nerve motor conduction velocity (m/s)	40.06 ± 4.21	53.37 ± 7.24	0.005
Superficial peroneal nerve SNAP amplitude (μV)	3.01 ± 1.07	5.56 ± 2.73	0.005
Superficial peroneal nerve sensory conduction velocity (m/s)	40.11 ± 3.03	47.32 ± 5.93	0.023
Superficial peroneal nerve CMAP amplitude (μV)	5.03 ± 2.15	6.01 ± 3.39	0.012
Superficial peroneal nerve motor conduction velocity (m/s)	44.73 ± 4.91	54.12 ± 6.09	0.01
Median nerve sensory conduction velocity (m/s)	53.77 ± 5.16	55.36 ± 5.95	0.08
Median nerve SNAP amplitude (μV)	9.25 ± 3.88	11.28 ± 5.06	0.034
Median nerve motor conduction velocity (m/s)	52.62 ± 4.41	56.15 ± 5.96	0.06
Median nerve CMAP amplitude (μV)	7.31 ± 2.08	9.06 ± 3.16	0.02
Ulnar nerve sensory conduction velocity (m/s)	51.07 ± 5.49	58.10 ± 6.44	0.032
Ulnar nerve SNAP amplitude (μV)	6.93 ± 2.01	8.36 ± 3.17	0.001
Ulnar nerve motor conduction velocity (m/s)	57.90 ± 6.25	61.32 ± 8.04	0.07
Ulnar nerve SNAP amplitude (μV)	6.78 ± 1.84	8.16 ± 2.48	0.022

Key: SNAP: sensory active nerve potential; CMAP: compound motor active potential; uMMAr: urinary methylmalonic acid/creatinine.

Table 4: The correlation analysis between urinary methylmalonic acid/creatinine and electrophysiological markers of neuropathy severity.

Electrophysiological markers of neuropathy severity	r	P
Sural nerve SNAP amplitude (μV)	−0.131	−0.06
Sural nerve sensory conduction velocity (m/s)	−0.120	−0.065
Sural nerve CMAP amplitude (μV)	−0.196	−0.02
Sural nerve motor conduction velocity (m/s)	−0.205	−0.01
Superficial peroneal nerve SNAP amplitude (μV)	−0.0034	−0.69
Superficial peroneal nerve sensory conduction velocity (m/s)	−0.496	−0.0005
Superficial peroneal nerve CMAP amplitude (μV)	−0.197	−0.015
Superficial peroneal nerve motor conduction velocity (m/s)	−0.327	−0.003
Median nerve sensory conduction velocity (m/s)	−0.115	−0.07
Median nerve SNAP amplitude (μV)	−0.089	−0.15
Median nerve motor conduction velocity (m/s)	−0.139	−0.055
Median nerve CMAP amplitude (μV)	−0.002	−0.86
Ulnar nerve sensory conduction velocity (m/s)	−0.187	−0.02
Ulnar nerve CMAP amplitude (μV)	−0.315	−0.005
Ulnar nerve motor conduction velocity (m/s)	−0.009	−0.60
Ulnar nerve SNAP amplitude (μV)	−0.125	−0.065

SNAP: sensory active nerve potential; CMAP: compound motor active potential.

amplitude ($r = -0.197$, $P = 0.015$), and ulnar nerve sensory conduction velocity ($r = -0.187$, $P = 0.02$) showed modest inverse correlations with uMMA/creatinine.

4. Discussion

Our data showed polyneuropathy was present in 24.7% of our diabetic patients using electromyogram (EMG), which was higher than the previous report [11], maybe because of sample selection or the diagnostic criteria of neuropathy. We demonstrated a significant association between polyneuropathy and duration of diabetes in our patients and no association with age, while, in the Knuiman and Young studies, neuropathy was correlated with age and duration of diabetes [12, 13]. This may reflect the age distribution (all were below 70 years of age in the study).

The bMMA level in the patients with Vit B12 deficiency was significantly higher than in the people with normal Vit B12, while there was no significant difference between the two groups with normal Vit B12, which meant bMMA was not sensitive for early vitamin B12 deficiency. It is not considered the gold standard for the determination of Vit B12 deficiency

[14], because its concentrations are also elevated in renal failure, thyroid disease, small-bowel bacterial overgrowth, and conditions of hemoconcentration [15].

In the study, holoTC gradually reduced accompanied with Vit B12 decrease. There was great difference among the three groups, and it was closely related to DPN. We thought that holoTC is a sensitive marker for Vit B12 deficiency, as previously reported [16]. HoloTC measurement has come of age; however, it has not acquired wide clinical acceptance to date, maybe due to the test's cost and limited availability [17].

The present results demonstrated that when part of Vit B12 was in normal range, uMMA had obviously exceeded the normal level, and there was great difference among the three groups, so we thought that uMMA was sensitive for the early deficiency of cobalamin, consistent with the previous reports [18, 19]. The fasting urinary MMA concentration corrected for creatinine was highly correlated with diabetic polyneuropathy. The mechanism was unclear, and the probable mechanism was that methylmalonic acid affected energy metabolism and the citric acid cycle and then led to neurotoxicity and the occurrence of DPN. In addition, uMMA was reported related to the enhanced inflammation in acute myocardial infarction [20]. The uMMA measurements are a noninvasive method that requires only a 1 mL urine specimen, and for months if frozen, it may offer a new method of analysis for identifying true tissue cobalamin deficiency [21].

The nerve conduction velocity and amplitude of four limbs decreased obviously as uMMA/creatinine was above normal. There was no significant difference between the decreased conduction velocity of median and ulnar nerves and the increased uMMA/creatinine. The upper limb sensory nerves appeared to be preferably affected by axonal damage as demonstrated by the finding that the sensory active nerve potential amplitude of the median and ulnar nerves was reduced in a remarkably high percentage of study patients, versus the conduction velocity of the two nerves [22]. The abnormality in the superficial peroneal and sural nerves in the lower limb was more severe than in the median and ulnar nerve. The presence of conduction block and abnormal amplitude in our patients also raises the possibility that it was a manifestation of a more widespread demyelinating polyneuropathy, according to [23]. The correlation analysis showed that the nerve conduction block of lower limbs was more serious in the group of the increasement of uMMA/creatinine.

Our findings were presented with some limitations. Although we identified patients prospectively, they were not randomly selected from a population with Type 2 diabetes with or without DPN. We underwent EMG for each patient. Being an invasive operation, EMG could not reflect early neuropathy screening, so some early neuropathy patients might be missed. We excluded the patients with Type 1 diabetes because of their expected metformin restriction and the distinct pathophysiological mechanisms, and further study would be conducted in depth.

In conclusion, uMMA and holoTC are significantly associated with cobalamin deficiency and polyneuropathy. The relationships among cobalamin deficiency, decline holoTC, elevated uMMA levels, and the polyneuropathy are controversial, and further work is needed to prove a direct causal relationship and the mechanism, but both cobalamin deficiency and elevation of its serum metabolites are associated with the presence of a sensorimotor peripheral neuropathy [24]. uMMA may exacerbate polyneuropathy as a result of other unknown mechanisms; a clear understanding of its role necessarily awaits further research on the pathogenesis of DPN. Despite these limitations, we believe that uMMA is a potential iatrogenic contributor to the severity of the polyneuropathy in the population described. It can be recommended that uMMA, alone and in combination with holoTC, has better diagnostic efficiency in diagnosing Vit B12 deficiency for the diabetic patients and is expected to become more sensitive predicting and monitoring indicators of diabetic polyneuropathy.

Conflict of Interests

The authors declare that they have no conflict of interests.

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