Journal of Diabetes Research

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Lead Guest Editor: Celestino Sardu
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Editorial Board

Contents

Hindawi
Journal of Diabetes Research
Volume 2019, Article ID 1905194, 2 pages
https://doi.org/10.1155/2019/1905194

Editorial

Diabetes Mellitus and Its Cardiovascular Complications: New Insights into an Old Disease

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Received 21 April 2019; Accepted 21 April 2019; Published 19 May 2019

There are ~415 million people living with diabetes mellitus worldwide, with type 2 diabetes (T2DM) accounting for more than 90% of diabetic patients [1, 2]. T2DM negatively affects the prognosis of patients by markedly increasing both hospitalization and mortality rate [1]. The common phenotype of T2DM is characterized by relative insulin deficiency caused by pancreatic β-cell dysfunction and insulin resistance in target organs [2–4]. These aspects eventually cause an altered glucose homeostasis, with consequent systemic negative effects on molecular and cellular functions [5–7]. Coming in the merit of the present editorial, we edited the Special Issue "Diabetes Mellitus and Its Cardiovascular Complications: New Insights into an Old Disease," collecting the state-of-the-art research in the field. Indeed, T2DM is a relevant cardiovascular (CV) risk that is known to be the leading cause of morbidity and mortality associated with T2DM [8]. Insulin resistance and hyperglycemia work together as continuous negative triggers impairing ionic channel activity, the epigenetic program, and the cellular function of several organs [8]. At the clinical level, T2DM is strongly associated with both micro- and macrovascular complications, including retinopathy, nephropathy, and neuropathy, as well as cerebrovascular disease, ischemic heart disease (IHD), and peripheral artery disease (PAD) [1, 9]. Several important concepts need to be highlighted: (1) diabetic nephropathy, cardiomyopathy, and PAD are frequently diagnosed at later disease stages; (2) screening programs are inconsistent and often inadequate to reduce the burden of these disorders [1]; (3) therefore, diet, exercise training, and lifestyle changes remain useful tools in preventing or at least delaying CV complications of T2DM [1, 10]. Remarkably, dysfunctional ionic channels can be detected in T2DM patients without structural heart disease by direct alterations of ionic currents [11] as well as in patients with concomitant heart failure (HF) [12]. T2DM might increase the risk of atherosclerosis [1] alongside with a loss of regenerative myocardial muscle functions during an acute coronary syndrome. Intriguingly, T2DM might also cause functional alterations in the absence of obstructive coronary stenosis [13]. Indeed, altered glucose homeostasis and insulin resistance might trigger an advanced atherosclerosis in coronary arteries in cases with obstructive coronary stenosis and also in patients with nonobstructive coronary stenosis [13, 14]. To date, T2DM has been shown to determine abnormalities in the dynamic responses to vasoactive stimuli, leading to increased rates of major adverse cardiac events (MACE) [13–15].On the other hand, T2DM might cause complex electrical alterations in HF patients increasing the risk of atrial and ventricular arrhythmias [16]. Specifically, T2DM induces alterations of ionic currents affecting action potential genesis and propagation in cardiac chambers, increasing automaticity and reentry mechanisms and favoring both atrial and ventricular arrhythmias [16]. Additionally, the increased inflammation and advanced cardiac fibrosis can lead to mechanical abnormalities of cardiac

muscle with severe pump failure as well as higher rate of congestive HF and hospital admissions for HF worsening [15, 16]. In this setting, new drug therapies such as interventional treatments have been developed to revert these negative conditions in order to ameliorate not only coronary and cardiac function but also clinical prognosis in IHD and HF patients with T2DM [14–16]. Moreover, there is an increasing necessity to develop new diagnostic tools for early detection of CV complications as well as new efficient treatments for this pathological condition. Thereby, a better understanding of specific diabetes genotypes and phenotypes might result in more specific and tailored management of T2DM patients [1]. Hence, there is an urgent need to find new therapeutic approaches to blunt the systemic and tissue-specific effects of hyperglycemia and insulin resistance and to reduce the development of diabetic CV complications. In conclusion, we believe that the main goal in the near future will be to find treatments better tailored to diabetic patients using a personalized-medicine approach.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Celestino Sardu
Claudio De Lucia
Markus Wallner
Gaetano Santulli

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Hindawi
Journal of Diabetes Research
Volume 2019, Article ID 5157024, 10 pages
https://doi.org/10.1155/2019/5157024

Research Article

The Role of the Autonomic Nervous System on Cardiac Rhythm during the Evolution of Diabetes Mellitus Using Heart Rate Variability as a Biomarker

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Received 13 July 2018; Revised 29 December 2018; Accepted 11 February 2019; Published 9 May 2019

Guest Editor: Celestino Sardu

Heart rate variability (HRV) is highly influenced by the Autonomic Nervous System (ANS). Several illnesses have been associated with changes in the ANS, thus altering the pattern of HRV. However, the variability of the heart rhythm is originated within the Sinus Atrial Node (SAN) which has its own variability. Still, although both oscillators produce HRV, the influence of the SAN on HRV has not yet been exhaustively studied. On the other hand, the complications of diabetes mellitus (DM), for instance, nephropathy, retinopathy, and neuropathy, increase cardiovascular morbidity and mortality. Traditionally, these complications are diagnosed only when the patient is already suffering from the negative symptoms these complications implicate. Consequently, it is of paramount importance to develop new techniques for early diagnosis prior to any deterioration on healthy patients. HRV has been proved to be a valuable, noninvasive clinical evidence for evaluating diseases and even for describing aging and behavior. In this study, several ECGs were recorded and their RR and PP intervals were analyzed to detect the interpotential interval (ii) of the SAN. Additionally, HRV reduction was quantified to identify alterations in the nervous system within the nodal tissue via measuring the SD1/SD2 ratio in a Poincaré plot. With 15 years of DM development, the data showed an age-dependent increase in HRV due to the axon retraction of ANS neurons from its effectors. In addition, these alterations modify the heart rhythm-producing fatal arrhythmias. Therefore, it is possible to avoid the consequences of DM identifying alterations in SAN previous to its symptomatic appearance. This could be used as an early diagnosis indicator.

1. Introduction

Heart rate variability (HRV) results from the interaction between the ANS and the SAN activity [1]. Measurements of the fluctuations within HRV are a noninvasive method used to evaluate the nervous system under physiological and pathological conditions [2]. Such fluctuations arise from the regulation between the sympathetic and parasympathetic nervous systems, branches of the ANS [3] which have been evaluated with spectral analysis and time series methods [4]. The time series analysis of HRV is considered to be a trustworthy biomarker to evaluate diseases and even for describing aging and behavior [5]. For DM, HRV is an early biomarker for determining the progression of the illness [6].

Arroyo-Carmona et al. [2] used the RR time series of several electrocardiograms (ECG) for defining the variability in HRV. An ECG is the record of the electrical activity of the heart tissue, each of which is represented by different waves with distinctive amplitudes and durations. The ECG morphology is the result of the ANS and SAN activities and can be classified into two groups: the positive deflections and the negative deflections. The positive deflections encompass the *P*, *R*, and *T* waves. The *P* wave represents the electrical activity of both atrial nodes, the *R* wave represents the ventricular depolarization, and the *T* wave represents ventricular repolarization [6]. The negative deflections include the *Q* and *S* waves. The most commonly taken into account intervals for measuring HRV are the *R*-*R*, *Q*-*T*, and *P*-*R* intervals and the QRS complex [7]. The pacemaker of the heart generates the electrical activity responsible for the intrinsic heart rate which is in the SAN. Although its depolarization cannot be seen on the ECG, the shape of the *P* wave could give an idea of its electrical behavior [8]. HRV has been thought to be solely the variability of the ANS and has been therefore statistically analyzed for serving only as a predictor of the regulation of ANS. However, as recent studies reveal that the SAN also has its own variability, it is of paramount importance to separately evaluate the correlation of both oscillators in order to use HRV to be an even better biomarker for evaluating diseases and even for describing physiological conditions such as aging and behavior. The aim of this study is to prove HRV as a clinical biomarker for framing the changes during the progression of DM. For this purpose, an animal model of chronic diabetes type 1 in mice (cDM) was used.

2. Material and Methods

2.1. Animal Model (Diabetes Mellitus Type 1). Adult male mice CD1 8 weeks old with 33 g of weight on average were used in this study. All the animals were maintained with a 12 : 12 h light-dark cycle (7:00-19:00) and allowed free access to LabDiet 5001 pellets and water. The cDM model was induced with streptozotocin at 120 mg/kg weight, and it was used thereafter at 10 and 20 weeks of induction DM (cDM model) [2]. All methods used in this study were approved by the Animal Care Committee of Instituto de Fisiología Celular, Universidad Nacional Autónoma de México. Animal care was in accordance with the "International Guiding Principles for Biomedical Research Involving Animals," Council for International Organizations of Medical Sciences, 2013 [2].

2.2. Diabetes Mellitus Evaluation: Electrocardiogram. The electrical activity was recorded at 8 weeks of age just before the DM induction; ten and twenty weeks following induction of DM, the parameters were compared with control. The mice were anesthetized with pentobarbital sodium 0.63 g/kg i.p. and placed in supine position for 30 minutes of ECG recordings. The bipolar ECGs were recorded with subcutaneous needle electrodes in configuration lead I. The electrodes were placed right and left in the fourth intercostal space. The ECG signal was amplified 700 times and filtered at 60 Hz. The signal was recorded on a PC at a sampling frequency of 1 KHz and analyzed offline with Clampfit® program (Molecular Devices). For the HRV analysis, the 30-minute long ECG recordings were cut into 5-minute series [7]. Subsequently, a hundred RR, PP, and action potential intervals were randomly selected. The intervals were measured between consecutive beats. All mice were continuously monitored to guarantee adequate ventilation and temperature.

2.2.1. Intrinsic Heart Rate Variability Recording of the Pacemaker. The nodal tissue was prepared as previously reported by Arroyo-Carmona et al. [2], and spontaneous electrical activity was recorded using the conventional microelectrode technique. The interpotential interval (ii) was measured for all zones of the pacemaker [3].

2.2.2. Heart Rate Variability Evaluation. For the evaluation of HRV, two approaches were used. The first was used to fit the tendency of the power spectral density (PSD), for determining the behavior of the time dependence within HRV. The second item was used for determining the magnitude of variability which was calculated SD1, SD2, and intrinsic heart rate variability using the Poincaré plot. For the construction of the Poincaré plot, the RR and PP intervals were used, which are the time between the maximum of the corresponding waves on the ECG and the interpotential interval of the pacemaker.

The Poincaré plot represents the RR_{i+1} interval as a function of the previous RR_i interval. The heart rate is the inverse RR interval. SD1 is the standard deviation of the distances between all points of the Poincaré diagram and the $RR_{i+1} = RR_i$ line. SD2 is the standard deviation of the distance between all points of the Poincaré diagram and the $RR_{i+1} = -RR_i + 2\overline{RR}_i$ line where $\overline{RR}_i$ is the average value of all RR_i [2]. iHRV is the SD1/SD2 ratio which is the value that suggests the delicate equilibrium between the sympathetic and parasympathetic systems of the heart [8]. Also, the Poincaré diagram was made with PP_i intervals and interpotentials (ii); the first reflected the auricular electrical activity. For the evaluation, the behavior of the whole correlation function used the power spectrum temporary time series RR and PP intervals of ECG of several stage ages of mice.

2.3. Data Analysis and Statistics

2.3.1. Poincaré Plot. All the data are presented as mean ± standard error. The *t*-test was used for data analysis; the values were considered statistically significant if the value was lower than 0.05 which is denoted with $*$. The analysis was made in the OriginPro version 8.0 from Lab Corporation.

The distances for the obtained SD1 and SD2 were calculated with

$$\sqrt{\left(\frac{RR_i - RR_{i+1}}{2}\right)^2}, \quad (1)$$

$$\sqrt{2\left(\frac{2\overline{RR}_i - RR_i - RR_{i+1}}{2}\right)^2}. \quad (2)$$

With all distances in equations (1) and (2), the SD1 and SD2 standard deviations were determined, respectively.

2.3.2. Power Spectral Analysis. In analyzing the frequency content of the signal $f(t)$, one might like to compute the ordinary Fourier transform $F(w)$; however, for many signals of interest, the Fourier transform does not formally exist. Because of this complication, one can work as well with a truncated Fourier transform where the signal is integrated only over a finite interval $[0, T]$:

$$F(w) = \frac{1}{\sqrt{T}} \int_0^T f(t) e^{-itw} dt. \quad (3)$$

This is the amplitude spectral density. Then, the power spectral density (PSD) can be defined as [2, 3]

$$S(\omega) = \lim_{T \to \infty} E\left[|F(w)|^2\right]. \quad (4)$$

By fitting the tendency of the PSD, it is possible to characterize the behavior of a system; for example, if $f(t)$ is a white noise signal (which is characterized for having all possible frequencies in the same fraction), the tendency will be a line with zero slope (m). Other examples consist in signals known as scale invariant which have a slope depending on the frequency (w) as $1/w$ [4].

2.3.3. Immunofluorescent Staining. Indirect immunostaining was analyzed using confocal microscopy (Confocal Olympus FV1000, Olympus America Inc.). SA nodes were isolated as mentioned above, embedded in Tissue-Tek (Sakura), frozen, and cut coronally into 5 μm thick slices beginning from the endocardium. The antibodies used were anti-tyrosine hydroxylase (1 : 250 rabbit polyclonal antibody; Millipore Corporation) and CY5 (1 : 200, rabbit polyclonal antibody; Jackson ImmunoResearch Laboratory Inc.).

3. Results

3.1. Development of the cDM Model Compared with Human. For relating ages between the animal model and human, a scale was constructed according to Dutta [9] and Koening [6]. Mouse adulthood ($n = 15$), as related to human age, is eight weeks compared with humans, which is at 17 to 22 years of age, according to Dutta [9]. Mice at eighteen weeks are 30 to 35 years old ($n = 13$) [9]; they must have 8 years of development with DM ten weeks after induction DM ($n = 13$). The mice at twenty-eight weeks are 40 to 45 years old ($n = 10$), and the DM model has chronic diabetes with 15 years development of DM twenty weeks after induction ($n = 13$).

3.2. Heart Rate. The heart rate was described using common RR intervals; in age three in control mice, the mean for adulthood (17-22 years human age) was 284 ± 46 bpm; the mean data showed an increase by 31%; at eighteen weeks or 30 years old and at twenty eight weeks old or 40 years old, the mice increased by 34% (Table 1). The heart rate decreased by 16% in early DM (ten weeks of development) compared with control and increased by 10%, beside adulthood. On the other side, the animals with chronic DM (twenty weeks of development) had an increase by 16% compared with control and 43% with adulthood (Table 1).

Table 1: Comparison of heart rhythm between age and development of DM.

Mouse age (weeks)	Human age (years)	DM development (human time)	BPM control	BPM (cDM)
8	17-22	—	284 ± 46	
18	30-35	8-10 years	371 ± 51$^{\infty}$	313 ± 78$^{\infty *}$
28	40-45	15 years	383 ± 64$^{\infty}$	405 ± 61$^{\infty *}$

DM was induced in 8-week-old mice; $^{*}\mu \pm$ SEM vs. control; $^{\infty}\mu \pm$ SEM vs. adulthood.

Also, the heart rate was characterized with PP intervals. In the same way, the heart rate increased with age; in adulthood, it was 279.06 bpm, 329.6 bpm at 30 years of age, and 379 bpm at 40 years of age. In the early eight years of development of diabetes, the heart rate decreased by 10% compared with control and did not change with adulthood. Subsequent of fifteen years of developing diabetes, the mice did not present changes compared with control animals, but compared with that at adulthood, the heart rate has an increase by 35%.

As expected, the pacemaker presented a low rate of firing by aging, and the frequency intrinsic at 30 years was 218 (ii/min) and 190 (ii/min) at 40 years old, inasmuch as the autonomic nervous system was unmodulated. The animals with diabetes in the early and chronic stages augmented rate firing at 258 and 208 respectively, although following the rule of decrease in firing by aging (see Table 2).

3.3. Heart Rate Variability

3.3.1. Heart Rate Variability. For analysis of HRV, we have studied two different types of time series, the PP and RR series obtained from the ECG. Each of them is from three control cases with adulthood, 30 and 40 years old, and two from a group of ill subjects with the same ages as in the control groups.

(1) The Poincaré Plot. In the Poincaré graph of RR intervals, during adulthood, variability of SD1 = 12, SD2 = 28, and ratio of 0.43 similar to humans was observed [10, 11]; the variability decreased by age, at eighteen weeks of age variability decreased to SD1 = 2, and at twenty-eight weeks of age variability was SD1 = 1, while SD2 only changed in the last age, SD2 = 1.3 (Table 3 and Figure 1). When using PP intervals for the Poincaré plot, the HRV decreased by age in both SD1 and SD2; the literature suggests for humans [1]. The HRV in adulthood was observed with SD1 = 19 and SD2 = 35 and ratio of 0.54; when the animals are 30 years old, they show a decrease of 60% and 32%, whereas the mice with 40 years of age have 1.1 and 1.1 for SD1 and SD2, respectively (Table 3 and Figure 1). Diabetes in the early stages altered the delicate equilibrium of the autonomic nervous system, while SD1 increased with 8 and 10 and SD2 with 46 and 56

Table 2: Alterations of HRV of frequency pacemaker by diabetes.

Frequency (ii/min)	Intrinsic activity (ms)	SD1	SD2	Poincaré index		
				SD1/SD2	Frequency	Variation CT vs. cDM
8 years' development						
CT = 218 ± 55	$Interval_{CT}$ = 275 ± 73	10	61	0.2		
cDM = 258 ± 50*	$Interval_{cDM}$ = 233 ± 50*	61*	45*	1.35	Increase 18%	Increase 377%
15 years' development						
CT = 190 ± 59	$Interval_{CT}$ = 351 ± 30$^{\infty}$	15$^{\infty}$	15$^{\infty}$	1		
cDM = 208 ± 63*	$Interval_{cDM}$ = 327 ± 23*$^{\infty}$	13*$^{\infty}$	14*$^{\infty}$	0.9	Increase 9%	—

Student t-test: $p < 0.05^{*}$ vs. control; Student t-test: $p < 0.05^{\infty}$ adulthood. ii: interval interpotential.

Table 3: Relationship HRV with age and development DM as human.

ECG (ms) interval	SD1	SD2	Poincaré index		
			SD1/SD2 ratio	Variation adulthood	Variation vs. control
Adulthood (17-22 old years)					
RR_{CT} = 218 ± 33	12	28	0.43	—	
PP_{CT} = 215 ± 45	19	35	0.54	—	
8 years' development					
RR_{CT} = 166 ± 28$^{\infty}$	2	28	0.07	Decrease 600%	
$RR_{cDM-model}$ = 206 ± 62* (24%)	8*	46*	0.17	Decrease 260%	Increase 256%
PP_{CT} = 182 ± 45	8.2$^{\infty}$	24$^{\infty}$	0.34$^{\infty}$	Decrease 68%	
$PP_{cDM-model}$ = 203* (12%)	10$^{\infty}$	56$^{\infty}$*	0.18*$^{\infty}$		Increase 188%
15 years' development					
RR_{CT} = 161 ± 30$^{\infty}$	1$^{\infty}$	1.3$^{\infty}$	0.8	Increase 86%	
$RR_{cDM-model}$ = 151 ± 23*$^{\infty}$ (24%)	0.6*$^{\infty}$	0.6*$^{\infty}$	1		Increase 25%
PP_{CT} = 158 ± 28	1.1$^{\infty}$	1.1$^{\infty}$	1$^{\infty\infty}$	Increase 232%	
$PP_{cDM-model}$ = 159 ± 29 (12%)	1.2$^{\infty}$	0.9$^{\infty}$	1.3*		Increase 30-%s

Student t-test: $p < 0.05^{*}$ vs. control, Student t-test: $p < 0.05^{\infty}$ adulthood.

in both system RR and PP intervals, respectively. When comparing the variability of the animal model with diabetes and adulthood, SD1 is low and SD2 rises (see Table 2 and Figure 2). After fifteen years of development of diabetes in mice, the variability decreases in RR intervals SD1 = 0.6, SD 2 = 0.6, and PP intervals SD1 = 1.2, SD2 = 0.9 in addition, SD1 and SD2 are similar, namely, nondynamic systems (Table 3 and Figure 2).

(2) The Power Spectral Density Analysis. In the PP time series, according to age change, it was observed that the tendency of the PSD increases [12, 13], which indicates alterations in the rigidity of the system, since it is favoring a specific frequency (Figures 3(d)–3(f)), which refers to the diabetes cases (Figure 4); although the slope is not zero, it is clearly closer to this value than in the control cases. This would mean that the heart is losing its characteristic frequencies and is approaching white noise (which has all frequencies indistinctly).

For the time series of RR of the control mice, in terms of age, nothing can be said with certainty, because the adjustment of the PSD has no order in the slopes (m); in fact, one of them is practically zero, which is what we would expect in case of diabetes [14] (see Figures 3 and 4). In cases of diabetes, there is a slope very close to zero, which reinforces the previous results (see Figure 4).

3.4. Heart Rate Variability of the Pacemaker. The HRV intrinsic of the pacemaker using interpotential intervals (ii) showed greater variability than all intervals, in adulthood SD1 = 58% and SD2 = 25% major than RR intervals; however, HRV decreased by age at 30 years old, SD1 = 67% and SD2 = 50%, and at age 40 years old SD1 = 1700% and SD2 = 3200% were decreased. The cDM model with 8 years development showed a decrease in SD1 = 48%, increase in SD2 = 60%, and with 15 years development a decrease in SD1 = 1600% and SD2 = 3800% (see Figures 1 and 3(e)–3(f) and Table 2).

In the same way, the Poincaré plot of the pacemaker showed an increase in the parasympathetic system SD1 = 61 and a concomitant lowering decrease in the sympathetic system SD2 = 45 at the 8-year development of DM compared with control SD1 = 10 and SD2 = 61, while the 15-year development of DM had an increase, SD1 = 13, SD 2 = 14, compared with control SD1 and SD2 15. The index

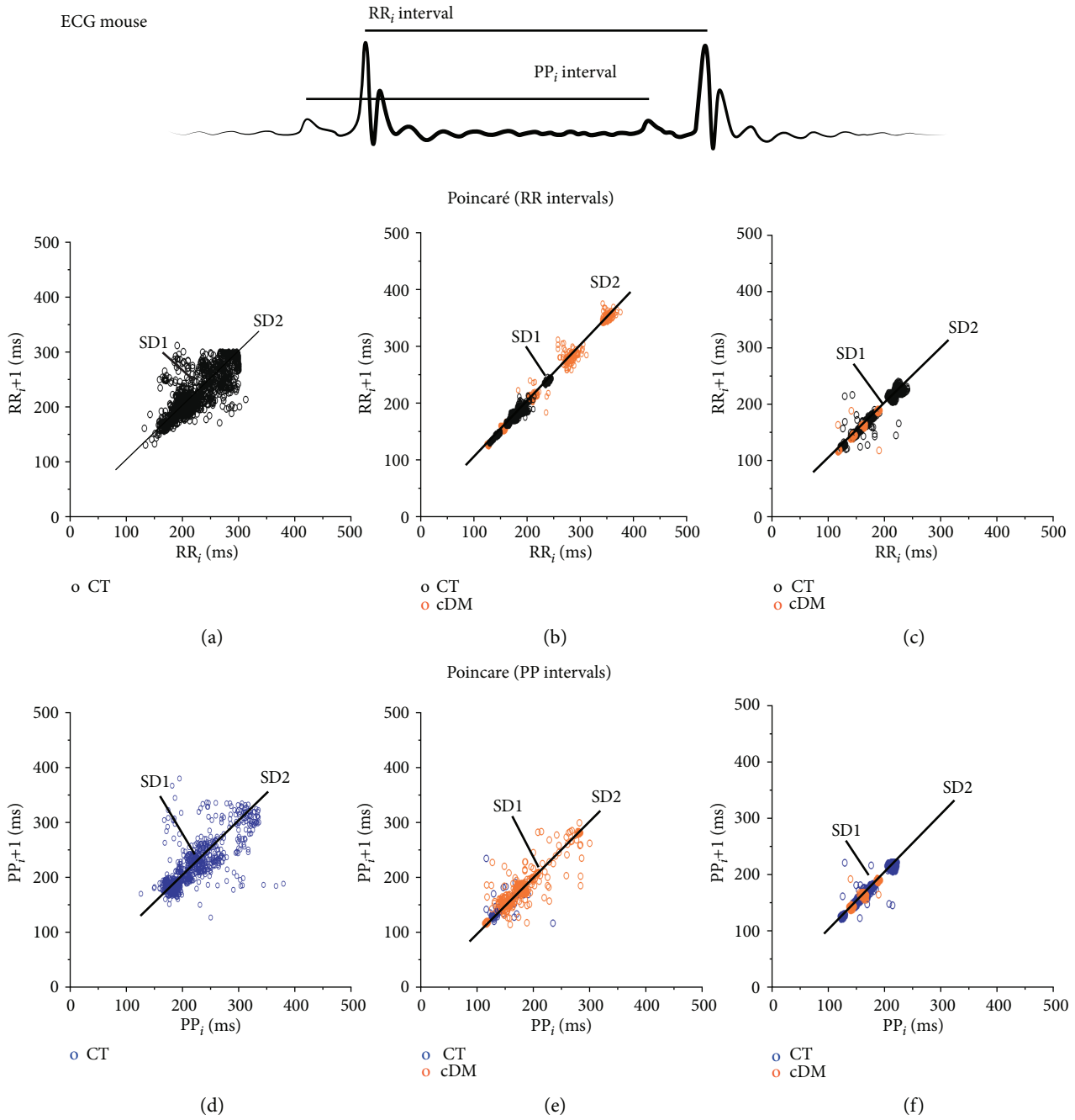

Figure 1: Age and development of diabetes mellitus alter the heart rate variability in the cDM model. (a, d) In adulthood, the morphology of the Poincaré plot is an ellipse with axe major SD2 and axe minor SD1, and the SD1/SD2 index was 0.5 for the PP interval and for RR interval SD1/SD2 = 0.43. (b, e) At 30 years of age with 8 years' development of DM, the data showed the function of the parasympathetic system. (c, f) At 40 years of age with 15 years' development of DM, the data showed that the nervous system ceased to function, and there were no changes in SD1 and SD2. Index with respect to control.

SD1/SD2 at 15 years of development was 1 and cDM 0.9 (Figure 2 and Table 2); this result was consistent with the decrease in tyrosine hydroxylase staining in the nodal tissue (Figure 5).

4. Discussion

DM is a higher factor of risk associated with cardiovascular mortality, in accordance with glucose management (diabetes mellitus type 1 or diabetes mellitus type 2) and other factors such as dyslipidemia, hypertension, microvascular complication, and duration DM [7]. However, the diagnosis for the DM type 2 is not timely; consequently, the poor glycemic control and combination with other factors could be manifest as tachycardia and development of "silent" myocardial infarction [15]. Furthermore, the telemonitoring of electrical activity of the pacemaker in patients at a very high risk developing fatal arrhythmias has helped to diminish

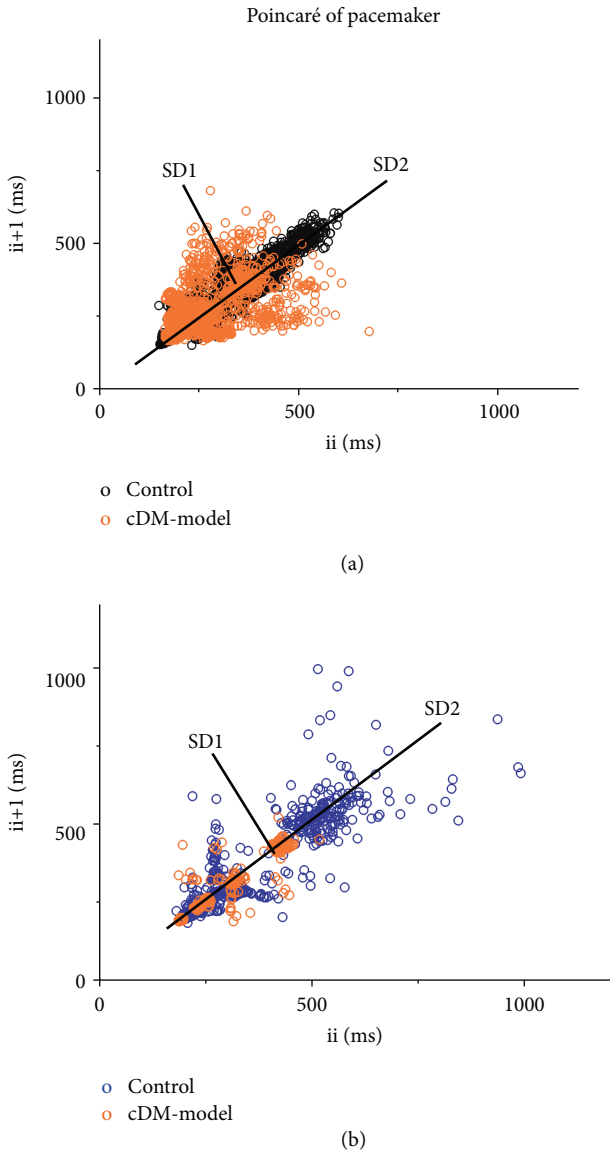

FIGURE 2: Pacemaker without the influence from the nervous system increases variability. The interpotential (ii) of the pacemaker in the control (a) at 30 years of age and (b) at 40 years of age. The development of diabetes mellitus increases both variability and frequency (a) at 8 years' development and (b) at 15 years' development of DM.

atrial fibrillation (AF) and ventricular tachycardia episodes significantly [16]; also in patients with pacemaker, it is a powerful diagnostic tool for predicting heart failure and reducing its hospitalization [17]. Thus, a method that is not invasive for diagnosis and prognosis is necessary, for lessening side effects as cardiovascular disease mortality. In this article, we proposed to use the interaction between ANS and SAN as a tool to inform above physiological and pathological conditions of body health.

The heart has electrical activity intrinsic with a physiological variability as an oscillator (Figure 2); SD1 represents the variability for a short time between the i-interval and the i-interval + 1 while SD2 is the variability of change with respect to average variability. The data in Tables 2 and 3 showed that pacemaker variability has an extensive range of frequencies to characterize its stable state; this means that the pacemaker variability could be modified by any perturbation outside its frequency range [3]. A physiological perturbation on the pacemaker is the ANS. In this case, for RR and PP intervals of ECG, it would be the interaction of the SAN-parasympathetic system as SD1, SAN-sympathetic as SD2, and SD1/SD2 as the relation between two oscillators (Figure 1). In the same way, the interaction between ANS and heart intrinsic activity is altered during aging similar to diabetes [18]; this involves fragility in the interaction between both SD1/SD2 (Tables 2 and 3).

The mice with early diabetes showed alterations in the delicate balance of the autonomic nervous system, such as SD1 decrease and SD2 increase added to resting tachycardia present in the pacemaker, suggesting cardiovascular autonomic neuropathy (CAN) in the early stages [19]. This data could be supporting the information about a poor diagnosis of diabetic autonomic neuropathy in early diabetic patients [20]. These patients may have only the silent AF as subclinical disease [21]. Other signs of a relationship with AT (atrial tachycardia) are changes in P-wave duration and dispersion [20]. This information proposed that the heart is the first organ injury for diabetes, the pacemaker primarily. Likewise, as diabetes progresses, the relationship with CAN is more evident. The mice with a fifteen-year development of diabetes showed resting tachycardia both in the heart with ANS and intrinsic pacemaker (Tables 1 and 3, respectively); additionally, these mice showed denervation in the pacemaker tissue (see Figure 5). The highest resting heart rate abnormality is related to damage in the parasympathetic system in the early stage of development of CAN [22].

A strategy to detecting CAN could be through reduction in HRV, measured by power spectral analysis; in healthy humans, beat-to-beat variation is recorded during inspiration and expiration, which is driven by sympathetic and parasympathetic activity to obtain three components of the power spectrum: (a) the thermoregulatory activity is reflected in very low frequency (0.003–0.04 Hz) or sympathetic activity; (b) the baroreceptor activity is reflected in low frequency (LF; 0.04–0.15 Hz) or a mixture of parasympathetic and sympathetic activity; and (c) it reflects respiratory activity expressed in high frequency (HF; 0.15–0.4 Hz) or parasympathetic activity [19, 22]. In this case, in the animal model, any cardiac autonomic cannot performed, and it has different component values in the frequencies compared to humans (see Figures 3 and 4).

In this paper, for measuring the reduction in HRV, the characterization in the behavior of the time dependence of PSD is proposed. The analyses of RR time series showed that the frequencies with major involvement in adulthood were 0.43 and 0.52 Hz; in mice with 30 years of age, the frequency is greater than 1.09 Hz; and at 40 years of age, the slope is near zero. The last point means that the time series is composite by all frequencies similar to a white noise (Figures 3(a)–3(c)). This suggests that the robustness of RR intervals decreases in the process of aging.

However, in the HRV of PP time series, the frequency major than 1.27 Hz was the biggest participation, a slope of

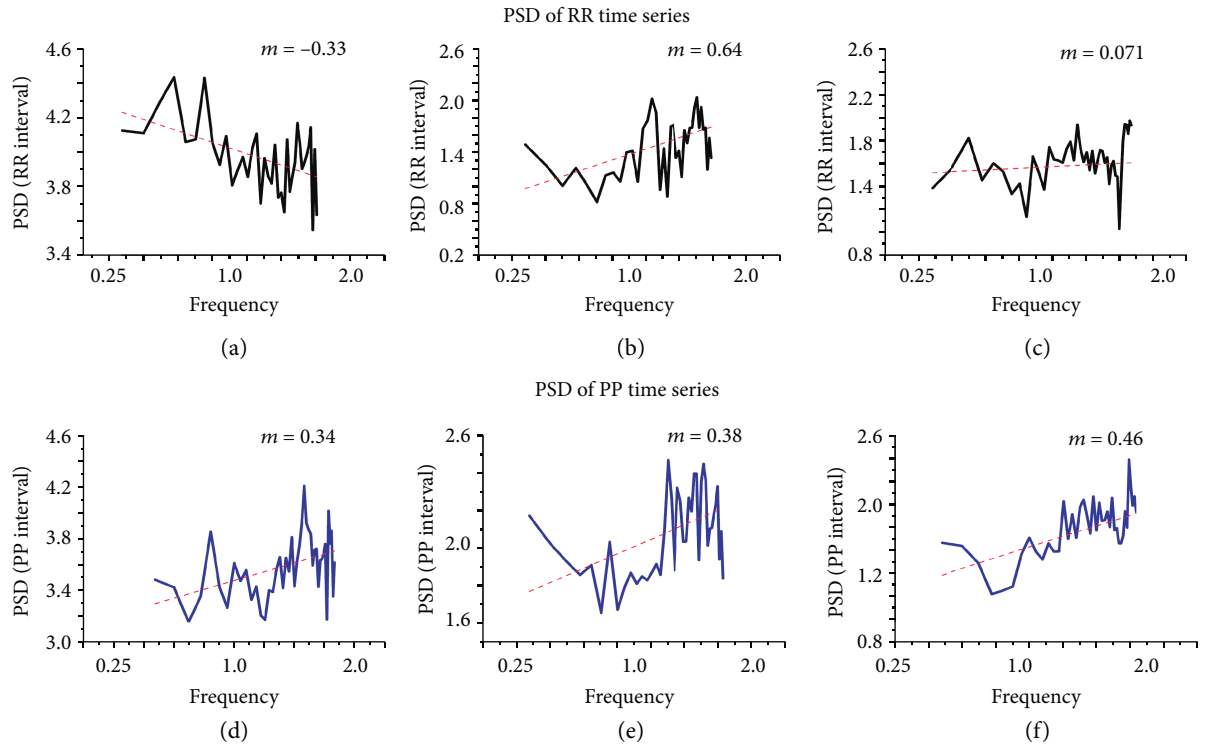

Figure 3: Power spectral analysis by age. Control subjects ($n = 15$) with increasing age from RR and PP time series. The slope varies with age with an erratic behavior in RR intervals (a, b, c). The slope of the fit line increases according to age in PP intervals (d, e, f).

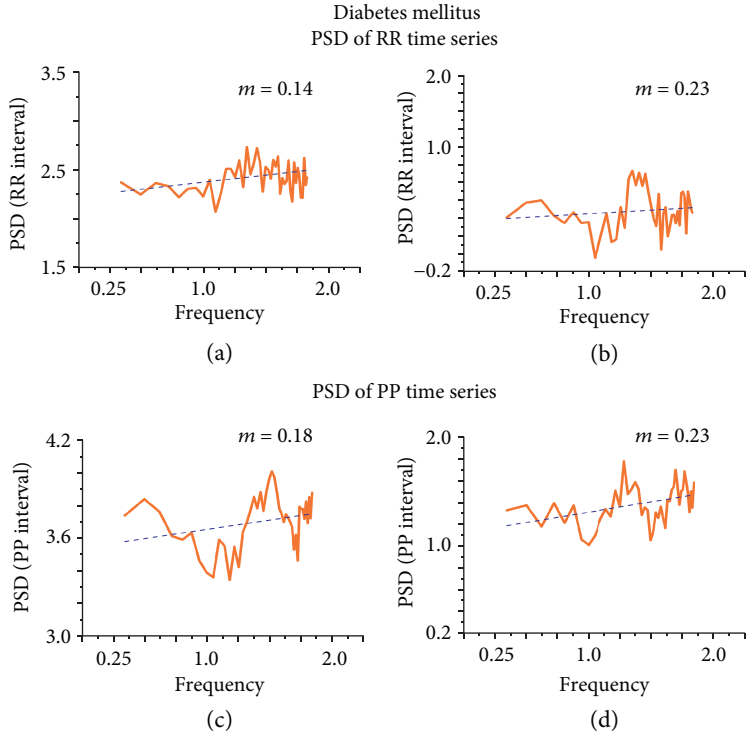

Figure 4: Power spectral analysis of the development of diabetes. The diabetes from RR time series (a, b) and PP time series (c, d) was a slope of approximately zero ($n = 13$) indicating loss of natural frequency PSD.

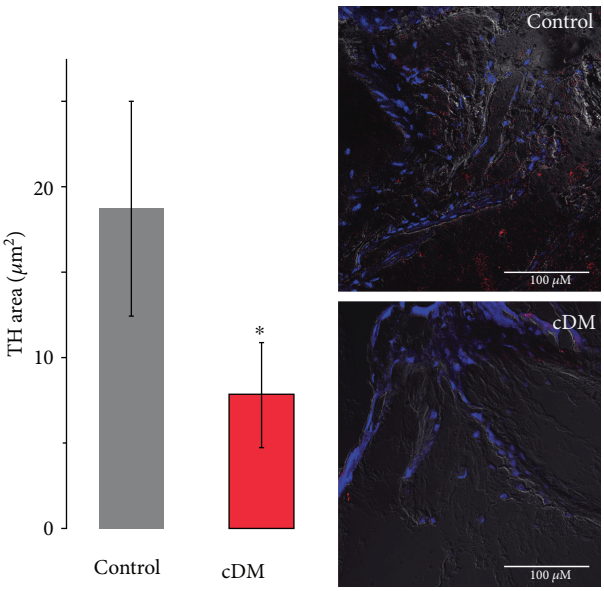

Figure 5: Decrease in the nervous system with 15 years' development of cDM. Average of nodal tissue staining with antibody of tyrosine hydroxylase (red) in the control and decreased signal in the nodal tissue of cDM mice ($n = 3$, Student t-test: $p < 0.05^*$ vs. control).

PSD was rising by age, and the analysis of PP time series allowed characterizing the frequencies during aging; for example, the slope is not zero (Figures 3(e) and 3(f)) [1]. The intrinsic activity or pp time series do not lose robustness.

In contrast, DM showed that both intervals (RR and PP) were white noise; this implies that the system loses robustness by diabetes (Figure 4). The characterization of HRV by this method is independent of maneuvers that implied to control more than one variable similar to thermoregulation, circadian rhythm, or respiration; in addition, several species could be used.

Also, HRV was quantified with a Poincaré plot analysis where the sympathetic-heart (SD2) interaction is double the parasympathetic and heart (SD1) interaction [23]; consequently, the Poincaré index was ~0.5 in adulthood (see Table 3). As age advances, this delicate relation decreases in SD1 but SD2 had changes after 30 years of age; this suggests that SD2 has a major participation in healthy conditions.

The results presented in alterations of HRV by early diabetic have been associated to the interaction lowering of the parasympathetic system and increase in the sympathetic system on electrical activity of the heart, without apparent shifts in the vascular system and peripheral nervous system (Figure 3). Chronic DM decreases by 20 times in SD1 and 46 times in SD2 with a ratio of 1 (see Figure 1 and Table 3). This could mean that the interaction between the ANS and heart was lost, so heart rate variability depends only the pacemaker (intrinsic activity) [3, 24] or the interaction between the sympathetic and parasympathetic systems is equal (autonomic balance) [25], such as the Poincaré plot of interpotential data which showed an index of 1 at fifteen years of DM (Table 2). According to the data shown in Figure 5, the nervous system of pacemaker tissue decreases in chronic diabetes and consequently increases the risk of CAN, such as the Poincaré plot of interpotential data which showed an index of 1 at fifteen years of DM (Table 3). According to the data shown in Figure 5, in the pacemaker tissue, the nervous system decreases in diabetes increasing the risk of CAN after fifteen years with DM. The variability of PP intervals allowed observing the sympathetic and parasympathetic systems' interaction due to aging and development of DM, such as the changes in SD1, SD2, and SD1/SD2 for aging SD1, SD2, and ratio decrease, while these parameters presented minor robustness in DM; on the other hand, the variability of RR intervals does not observe this correlation, explicitly with RR interval variability which does not sense changes in SD2 by aging (see Table 2).

It is known that dyslipidemias rise in the nervous system in SAN [3]. Thus, our cDM model may also be attractive for researches with new pharmacological treatments like GLP-1 and defibrillator [26]; both treatments could be anticipated of heart failure with dyslipidemia in the first stage of diabetes, decrease hospital admission and death in diabetic patients [16], and reduce microvascular complications [24]. For this reason, the development of an animal model like cDM with pharmacological chronic therapy of GLP-1 and monitoring your HRV would improve macro- and microvascular side effects inclusive of fatal cardiovascular events [27].

5. Conclusion

In the early stages of development of DM, the influence of the nervous system allowed maintaining the balance of an elliptical shape in the Poincaré plot; however, in diabetes mellitus by 15 years of development in SAN, this balance is altered in the PP interval Poincaré plot and PSD. However, in SAN, there is an increase in the variability at 8 and 15 years' development of DM. Therefore, it is important to observe the variability in PP intervals and increase changes in the rhythm and cardiac arrhythmias; finally, the proposal to use HRV for diagnostic and prognostic side effects by alterations in the rhythm producing fatal arrhythmias is very useful. It is also important that the PP interval is more useful as a diagnostic indication for diabetes than the RR interval is.

5.1. Clinical Implications. The analysis PP intervals of the cDM model showed the alterations of ANS preventing side effects and would allow diagnosis in several stages of DM patients. The data analyzed with this method infer the development of this disease with SD1, SD2, and SD1/SD2 of heart rate variability. Additionally, with analysis of PP intervals in PSD, this showed possibility of diagnosis and early prognosis of CAN. For this reason, we propose the use of HRV for diagnosis of DM chronic in several stages; additionally, it is a noninvasive and cheap tool and has easy arithmetic calculus.

Data Availability

The time series of ECG (PP, RR) and the nodal electrical activity (interpotential) data used to support the findings of this study are available from the corresponding author upon request to the email of Julián Torres-Jácome, PhD: jtorresjacome@gmail.com.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

AIA, ACRE, SHR, and ROG designed these experiments; AIA, DGG, and ACRE took responsibility for the data collection and ACRE, AIA, AF, DGG, and TJJ for the analysis of the data. All authors contributed to the drafting or revising of the manuscript, and all authors approved the final version of the manuscript.

Acknowledgments

This study was supported in part by Grants from project CONACYT, VIEP-BUAP 100059822 to JTJ, CONACYT through Fronteras grant FC-2016-1/2277, and the Universidad Nacional Autónoma de México through DGAPA-PAPI IT IV100116 and VIEP-BUAP 100500599 to REAC. We thank Myrian Velasco, Ph.D., for the helpful discussion. For expert technical support at the SERVALAB® Laboratory, S. Daniela Rodriguez Montaño IFC-Histología, UNAM, and MVZ Hector Alfonso Malagon Rivero IFC-Bioterio, UNAM, are acknowledged.

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Hindawi
Journal of Diabetes Research
Volume 2019, Article ID 1365162, 12 pages
https://doi.org/10.1155/2019/1365162

Research Article

Predictors of Effectiveness of Glucagon-Like Peptide-1 Receptor Agonist Therapy in Patients with Type 2 Diabetes and Obesity

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Received 12 July 2018; Revised 3 December 2018; Accepted 19 December 2018; Published 3 March 2019

Guest Editor: Celestino Sardu

Rationale. It is well known that diabetes mellitus (DM) exacerbates the mechanisms underlying atherosclerosis. Currently, glucagon-like peptide-1 receptor agonists (aGLP-1) have one of the most prominent cardioprotective effects among the antidiabetic agents. However, the treatment with aGLP-1 is effective only in 50-70% of the cases. Taking into account the high cost of these medications, discovery of the predictors of optimal response to treatment is required. *Purpose*. To identify the predictors of the greater impact of aGLP-1 on HbA1c levels, weight reduction, and improvement in lipid profile. *Methods*. The study group consisted of 40 patients with type 2 DM (T2DM) and obesity who were treated with aGLP-1. The follow-up period was 24 weeks. Patients' evaluation was conducted at baseline and after 24 weeks. In addition, it included the assessment of the hormones involved in glucose and lipid metabolism and appetite regulation. *Results*. Patients who have initially higher BMI (body mass index), glycemia, and triglycerides (TG) had better improvement in these parameters undergoing aGLP-1 treatment. In patients with a BMI loss ≥ 5%, GLP-1 and fasting ghrelin levels were higher and ghrelin level in postnutritional status was lower. The HbA1c levels decreased more intensively in participants with higher GLP-1 levels. TG responders had lower baseline fasting glucose-dependent insulinotropic peptide (GIP) and postprandial ghrelin levels. *Conclusion*. The evaluation of the glycemic control, lipid profile, and GLP-1, GIP, and ghrelin levels are useable for estimating the expected efficacy of aGLP-1.

1. Introduction

It is well known that the rates of mortality due to cardiovascular and cerebrovascular diseases are markedly higher among people with type 2 diabetes mellitus (T2DM) [1]. Currently, the underlying mechanisms that cause T2DM and increase cardiovascular diseases (in patients with T2DM) are believed to include abnormalities in the effects of incretins and other hormones involved in glucose metabolism and food intake regulation [2].

The incretin hormones—intestinal peptide hormones, the most widely studied of which are glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP)—are normally secreted in response to the oral ingestion of nutrients [2]. GLP-1 has a number of functions: augmenting insulin's response to glucose, slowing gastric emptying, and suppressing the secretion of glucagon. The latter activates the secretion of hepatic glucose and increases satiety.

GLP-1 receptor agonists (aGLP-1) have more prominent cardioprotective effects among the incretin-based antidiabetic agents. aGLP-1 demonstrate an ability to improve the prognosis for cardiovascular diseases by means of a decrease of atherosclerotic events [3]. They improve the prognosis in diabetic patients with myocardial infarction [4, 5] and may reduce arrhythmic burden and hospital admissions for heart failure worsening in diabetic patients [6].

A number of clinical studies show that aGLP-1 therapy results in a glycosylated hemoglobin (HbA1c) level reduction from 0.9 to 1.6% [7–12] and a body weight loss ranging from 0.2 to 7.2 kg [13]. The effective (target) decline of HbA1c and body weight is not observed in all patients.

The group of patients with good response does not exceed 50-60% averagely [12]. Taking into account the high cost of these medications, the identification of treatment response predictors is required.

According to the literature data, the initial high level of glycemia is considered to be one of the most significant predictors of a glucose-lowering effect of aGLP-1 [14].

In addition to the influence on carbohydrate metabolism and body weight, there is an aGLP-1 effect on other cardiovascular risk factors, particularly on blood pressure (BP) [15].

According to our previous research, there was also a more significant downturn in BP in patients receiving aGLP-1 with higher degrees of hypertension, while there was no correlation between weight loss and the decrease in BP [16]. These data have shown that aGLP-1 effects on BP regulation are independent from the weight loss mechanisms.

Moreover, aGLP-1 therapy leads to favorable changes in the lipid profile and other atherogenic risk factors [17–20]. aGLP-1 therapy positively modulates inflammation in atherosclerosis in diabetic patients. [21]. The dose-dependent decline in the levels of high-sensitivity C-reactive protein (hs-CRP), plasminogen activator inhibitor 1, B-type natriuretic peptide, and endothelin-1 was demonstrated in several studies [18–20]. It is important to note that the 65% reduction in hs-CRP levels was independent on the dynamics of body and fat mass [20]. These results imply that the anti-inflammatory and potential antiatherosclerotic effects of aGLP-1 are not always associated with weight loss effects [20].

The role of the hormones involved in glucose and lipid metabolism as regard to predicting the aGLP-1 therapy efficacy is not currently clarified. Incretins, ghrelin, leptin, and adiponectin are related to this group of hormones. Consequently, the study of these hormones is also of great interest.

In obese patients, the ghrelin level in fasting plasma is lower, but the reduction in its level after partaking food is not sufficient, in comparison to adults with normal body mass index (BMI) [22]. It is probably due to an adaptive reaction in response to a positive energy balance. The administration of GLP-1 or aGLP-1 leads to ghrelin level reduction [23–25] through the neuronal mechanisms involving the hypothalamus and peripheral nervous system [25, 26]. In contrast, there is a positive correlation between the adipose tissue mass and the level of fasting plasma leptin and free leptin index. However, the negative correlation between the soluble leptin receptor and body weight was demonstrated [27]. Recent studies have shown that GLP-1 partially inhibits appetite through the regulation of the amount of soluble leptin receptors [28]. Thus, the introduction of aGLP-1 inhibits growth in soluble leptin receptors induced by weight loss, thereby maintaining the level of free leptin and preventing weight gain [27]. One year liraglutide administration to patients who previously have lost more than 12% of body weight through the hypocaloric diet triggers greater weight loss, compared to that in the control group [27]. It may be due to the reduction of leptin resistance. However, the predictive role of orexigenic and anorexigenic hormone levels in treatment efficacy has not been established so far. A lower adiponectin level has been observed in obese and diabetic populations, suggesting a reverse correlation between the adiponectin level and insulin sensitivity [29]. It has been demonstrated that aGLP-1 increase adiponectin expression and secretion.

Thus, aGLP-1 effects on hormones involved in glucose and lipid metabolism and food intake regulation are explored to a certain extent. By contrast, the association between the hormone levels and effectiveness of aGLP-1 treatment seems to be underinvestigated.

Another potential factor influencing the efficacy of aGLP-1 treatment is food behavior type. Data from Dutch researchers show that patients with an emotional type of eating behavior are less sensitive to the central effects of exenatide [30]. In a study with obese patients with T2DM who received aGLP-1 during 2 years, body mass reduction depended on the eating behavior group. The external eating behavior ($n = 17$) resulted in the smallest weight loss and the restrained eating behavior resulted in the greatest one. Meanwhile, participants with emotional and indifferent eating behavior models showed average results [31]. According to our earlier results, patients with a restrictive type of eating behavior had a tendency to a greater decline in body weight in comparison to patients with combinations of two or three behavior types [32].

Thus, aGLP-1 provide the reduction of the following cardiovascular risk factors: weight excess, hyperglycemia, hypertension, and atherogenic lipid level. The dynamics of these factors can be determined by certain predictors, and identifying them is a crucial task. The aim of our study was to identify predictors of the response to aGLP-1 therapy with regard to the reduction of blood glucose level, weight, and effect on other metabolic parameters in patients with T2DM and obesity.

2. Methods

2.1. Participants. Initially, 44 patients who met the following inclusion criteria were recruited: (i) men and women at the ages of 18 to 75 years old, with an established diagnosis of T2DM, and who signed a patient informed consent form; (ii) the presence of obesity with BMI 30 kg/m^2 or more; and (iii) treatment with the following combinations of sugar-lowering agents: (a) biguanides, (b) biguanides + sulfonylurea medications, (c) biguanides + insulin, and (d) stable doses of glucose-lowering agents and/or hypolipidemic and antihypertensive drugs for a minimum of 3 months before inclusion.

Exclusion criteria include patients with the following conditions: (i) uncompensated hypothyroidism and/or endogenous hypercorticism; (ii) severe diseases of the cardiovascular system (congestive heart failure III-IV f. cl., uncontrolled hypertension, myocardial infarction, or acute cardiovascular event during the last 6 months); (iii) severe hepatic impairment and/or severe chronic kidney disease (C3a-C5); (iv) mental illness (including bulimia); (v) acute infectious diseases; (vi) exacerbation of chronic diseases; (vii) steroid therapy and (viii) any of the six aGLP-1 therapy contraindications to the treatment with aGLP-1 including (a) hypersensitivity, (b) diabetes mellitus type 1, (c) diabetic ketoacidosis, (d) pancreatitis in

anamnesis, (e) creatinine clearance < 30 ml/min, and (f) severe gastrointestinal diseases with gastroparesis.

13 men and 31 women were recruited in the study. All participants were prescribed the following aGLP-1 treatments: 14 patients with exenatide and 30 patients with liraglutide. The follow-up period was 24 weeks. 4 patients prematurely stopped participating in the study (one patient due to acute pancreatitis development and three patients due to the lack of finances to purchase the aGLP-1 medications). The final analysis included 40 patients: 12 men (30%) and 28 women (70%); 12 on exenatide (30%) and 28 on liraglutide (70%).

87.5% of the patients were taking antihypertensive therapy, and 57.5% of the patients were taking hypolipidemic therapy. The proportions of drug groups are as follows: β-blockers—37.5%, sartans—45%, angiotensin-converting enzyme inhibitors—35%, diuretics—50%, calcium channel blockers—27.5%, imidozolin receptor agonists—10%, statins—57.5%, fibrates—2.5%, and omega-3 polyunsaturated fatty acids—2.5%. During the follow-up period there were no changes in therapy.

2.2. Study Design

2.2.1. Visits Included in the Study

(1) A screening visit involved the following activities:

(i) Elimination of endogenous hypercorticism, uncompensated hypothyroidism, and other secondary causes of obesity

(ii) Compliance with the inclusion criteria check; individuals who met eligibility criteria were enrolled in the study and signed an informed consent

(iii) Giving the instructions to standardize antidiabetic, lipid-lowering, and antihypertensive therapy

(2) Visit 1 (at baseline) was conducted at 3 months after therapy standardization; patients started receiving treatment with aGLP-1 (12 patients with exenatide and 28 patients with liraglutide) according to the standard regimen

(3) Visit 2 was a follow-up conducted 24 weeks after treatment initiation. Parameters estimated at all study visits are listed in Table 1

Table 1: Estimated parameters.

Parameters/visits	Screening	Visit 1 at baseline	Visit 2 after 24 months
Anthropometric parameters	+	+	+
Vital signs	+	+	+
Questionnaire survey		+	+
Adiponectin		+	+
GIP		+	+
Leptin		+	+
Ghrelin fasting		+	+
Ghrelin after test*		+	+
GLP-1		+	+
HOMA-IR and HOMA-β		+	+
C-peptide fasting		+	+
C-peptide after meal test*		+	+
Lipid profile		+	+
HbA1c		+	+
Review of the therapy	+	+	+

*2 hours after breakfast in the meal tolerance test.

2.2.2. Control Group. There are no reference ranges for GLP-1 and GIP levels. Thus, we compared GLP-1 and GIP levels in the study sample with those of the group of healthy blood donors. The control group included 19 age- and gender-matched healthy blood donors (5 men (26.3%), 14 women (73.7%), without obesity and diabetes mellitus: BMI between 22 and 25 kg/m^2, fasting glucose level < 6.0 mmol/l, with a mean age of 48.6 years (from 26 to 65)).

2.2.3. Assessments. All patients underwent an initial assessment which included a survey (complaints, medical history), physical examination with the measurement of anthropometric parameters (height, body weight, BMI calculation, and waist circumference (WC)) and vital signs (BP, heart rate (HR), and respiratory rate (RR)), and completion of the questionnaire. The BMI calculation was carried out according to the formula $\text{body mass (kg)}/(\text{height (m)})^2$. Measurement of BP and HR was conducted after 5 minutes of rest, with patients seated. BP was measured 2 times with an interval of 5 minutes, and average level was recorded.

Patient's attitude to the food was assessed using two questionnaires:

(1) To assess the nature of nutrition, the Dutch questionnaire of food behavior DEBQ (Dutch Eating Behavior Questionnaire) was used

(2) Ratings of hunger, fullness, desire to eat, and perspective consumption were assessed using a 100 mm visual analogue scale at the fasting state (state visual analog scale to register the sensations of appetite—VAS fasting state)

The following laboratory methods were used in the study: the lipid profile was measured by the enzyme method (Roche, Germany); the level of HbA1c was detected by the method of affinity chromatography (Bio-Rad, USA); insulin level was determined using reagents and the COBAS INTEGRA 400 plus analyzer from Roche, France; levels of the hormones involved in appetite regulation and carbohydrate metabolism were evaluated using enzyme immunoassay methods for ghrelin (RayBiotech Test System (USA), leptin

and GLP-1 (ARCHITECT i1000SR analyzer from Abbott (USA)), adiponectin (BioVendor), GIP (ELISA Kit, Cloud-Clone Corp.), and C-peptide (Elecsys 2010 Analyzer).

The evaluation of the degree of insulin resistance with the determination of the HOMA index of insulin resistance (HOMA-IR) was calculated according to the following formula: fasting glucose (mmol/l) × fasting insulin (mcIU/ml)/22.5. To estimate the functional reserve of β-cells we used an index HOMA-β that is calculated by the following formula: 20 × fasting insulin (mcIU/ml)/(fasting glucose (mmol/l) − 3.5). For β-cell function assessment, we used the meal tolerance test based on the initial level and C-peptide level determined 2 hours after the ingestion of a standard breakfast. The nutrient solution for the breakfast consisted of 190 ml of pure water and 80 g of the Clinutren Optimum mixture for enteral nutrition.

2.2.4. Characteristics of the Study Group at Baseline. 40 patients were finally included in the study, including 12 men (30%) and 28 women (70%), with an average age of 57.1 years (from 27 to 75) and mean diabetes duration of 11 ± 7.2 years.

All indicators were assessed at baseline and after 24 weeks of treatment. Table 2 shows the clinical characteristics at baseline. BMI, WC, HbA1c, and glucose fasting levels were higher than normal ranges and corresponded with the criteria of diabetes and obesity. HOMA-IR exceeded the normal range more than two-fold. C-peptide level was normal in all patients and the mean value was in the middle of the normal range. After breakfast (2 h) in the meal tolerance test, an adequate growth in the C-peptide level (>50%) was achieved only in 66.7% of the participants. Most of the lipid profile components also differed from the target level for patients with diabetes. The mean total cholesterol (TC) and low-density lipoprotein (LDL) levels, the median for TG was higher and the median for high-density lipoprotein (HDL) among women was lower. However, the median for HDL among men was within target ranges. Mean systolic blood pressure (SBP) and diastolic blood pressure (DBP) levels were lower than the upper limit of normal; 42.5% of the participants had SBP > 140 mm Hg or/and DBP > 85 mm Hg.

Initial hormonal status is presented in Table 3. The initial adiponectin levels for both men and women were within the reference range, but they were less than the mean level of adiponectin (20.1 mcg/l) among healthy nonobese people in Saint Petersburg [33]. The initial leptin level for both genders was higher than the normal ranges. Fasting ghrelin level at baseline significantly differed from the reference ranges for healthy people: the mean value was more than four-fold below the lower limit of normal. After breakfast (2 h) in the meal tolerance test, an adequate downturn in ghrelin level (decrease by 30-55%) was achieved only in 40% of the participants. GLP-1 and GIP levels were compared with the control group, because there are no reference ranges for these hormones. A significant difference was observed in GLP-1 and GIP levels: in the control group these hormones were higher (Table 3(b)).

2.3. Statistical Analysis. Statistical analysis was performed using the program IBM SPSS Statistics 23 (Statistical Package for Social Sciences) and Statistica V. 7.0. Data are presented as numbers and percentage. Variables with a normal distribution are presented as mean ± SD and otherwise as median and interquartile range. The nonparametric Wilcoxon criterion was used to estimate the differences between dependent samples. With the help of the Mann–Whitney rank criterion, the reliability of the differences of independent variables was evaluated. The analysis of the relationship (correlation) of two quantitative traits was carried out by Spearman's test. The comparison between the different groups was carried out using a single-factor dispersion analysis (ANOVA) using a posteriori Student-Newman-Keuls criterion. For comparison of nominal variables, we used the Chi-squared test (Fisher's exact test). The differences were considered significant at $P < 0.050$.

Data in the tables are presented as mean ± standard deviation or median (25 percentile; 75 percentile).

2.4. Ethical Review. The research was approved by the Ethics Committee of the Almazov National Medical Research Centre (record no. 63, 14.04.14).

3. Results

We compared the mean reduction of BMI and HbA1c in the exenatide and liraglutide groups separately, but these parameters did not differ statistically significantly before and after treatment (Table 4).

Therefore, the results obtained in these groups were summarized.

3.1. What Has Changed after Treatment with aGLP-1? Table 5 shows the clinical characteristics at baseline and after 24 weeks of treatment with aGLP-1. By the end of the therapy period (24 weeks), BMI fell by 2.4 kg/m^2 ($P < 0.001$) and waist circumference by 6.6 cm ($P < 0.001$). Also, an improvement in carbohydrate metabolism parameters is seen: HbA1c decreases by 1.0% ($P < 0.001$) and fasting plasma glucose by 2.0 mmol/l, ($P = 0.004$).

To reveal the predictors of effective body weight loss and HbA1c reduction, we have divided the study sample into the following two subgroups: responders to the aGLP-1 treatment and nonresponders. Only body weight loss ≥ 5% from the initial level and reduction ≥ 1% in HbA1c have been considered as effective. Those subjects who showed these results were accepted as responders.

An effective body weight loss (≥5% from initial) was observed in 51.5% of the participants, and target HbA1c reduction (≥1%) was observed in 39.4% of the patients. Good treatment response as BMI reduction did not always coincide with target HbA1c reduction: among patients with effective BMI loss HbA1c decline < 1% was observed in 29.4%, and among participants with effective HbA1c reduction 7.7% were nonresponders in BMI loss. But in most cases, BMI and HbA1c reduction concurred: in the group with HbA1c reduction < 1%, only 26.3% of patients had a target BMI loss; in the group with HbA1c reduction between 1% and 2% and in the group with HbA1c reduction more than 2%, 85.7% of patients subsequently had a target BMI loss (Figure 1).

Table 2: Characteristics of the participants at baseline.

Characteristics	Patients on the aGLP-1, $n = 40$	Reference range
Age (years)	27-75 (57.1)*	NA
Diabetes duration (years)	11 ± 7.2	NA
Body mass (kg)	114.5 ± 26.4	NA
BMI (kg/m^2)	40.9 ± 7.6	<25
WC (cm)		
Men	125.0 (116.0; 148.0)	<90
Women	116.0 (104.8; 132.5)	<84
HbA1c (%)	8.4 ± 1.3	<6.5
Glucose fasting (mmol/l)	8.9 ± 1.8	3.3-6.1
C-peptide fasting (ng/ml)	3.5 ± 1.8	0.78-5.19
C-peptide after test** (ng/ml)	5.0 ± 2.8	>9.9 (↑ on >50% from baseline)
HOMA-IR	7.2 ± 4.4	<2.77
HOMA-β (%)	35.7 (24.5; 61.0)	≥100
TC (mmol/l)	5.2 ± 1.3	<4.5
HDL (mmol/l)		
Men	1.01 (0.79; 1.13)	>1.0
Women	1.07 (0.8; 1.3)	>1.2
LDL (mmol/l)	3.1 ± 1.1	<1.8
TG (mmol/l)	1.9 (1.5; 3.2)	<1.7
SBP (mm Hg)	138.7 ± 14.7	<140
DBP (mm Hg)	84.5 ± 8.4	<85

*Min-max (mean age). **2 hours after breakfast in the meal tolerance test.

Table 3

(a) Hormone levels in the study group at baseline: comparison with reference range

Hormones	Patients on the aGLP-1, $n = 32$	Reference range
Adiponectin (mcg/ml)		
Men	8.9 (6.8; 17.1)	>6
Women	10.1 (8.2; 13.8)	9-12
Leptin (ng/ml)		
Men	45.0 (8.0; 79.0)	0.5-13.8
Women	32.5 (13.5; 52.5)	1.1-27.6
Ghrelin fasting (pg/ml)	1.6 (0.8; 2.7)	8.502-16.6
Ghrelin after test* (pg/ml)	2.7 (1.4; 3.5)	↓ 25-55% from baseline**

*2 hours after breakfast in the meal tolerance test. **See reference [34].

(b) Hormone levels in the study group at baseline: comparison with control group

Hormones	Patients on aGLP-1, $n = 32$	Control group, $n = 19$
GLP-1 fasting (mmol/l)	0.07 (0.01; 0.14)	0.17 (0.12; 0.27)*
GIP (pg/ml)	391.6 (326.6; 461.4)	574 (520.4; 744.5)**

*$P = 0.003$. **$P = 0.0001$.

3.1.1. Blood Pressure and Lipid Profile. Among the characteristics of the cardiovascular system, SBP fell by 9.1 mm Hg ($P = 0.001$) and DBP by 5.8 mm Hg ($P = 0.001$). Lipid profile dynamic analysis revealed no significant changes. Similarly, there were no significant changes in C-peptide, HOMA-IR, and HOMA-β levels. The results are also presented in Table 5.

Table 4: Dynamics of body mass index and glycosylated haemoglobin in exenatide and liraglutide groups.

Therapy	BMI at baseline	BMI after 24 weeks	BMI reduction	Mean reduction of initial (%)	HbA1c at baseline	HbA1c after 24 weeks	HbA1c reduction	Mean reduction of initial (%)
Exenatide, $n = 12$	42.8	40.6	2.2	5.4	8.8	7.8	1.0	1.2
Liraglutide, $n = 28$	40.1	37.6	2.5	4.0	8.3	7.2	1.1	0.9
P-level				0.256				0.403

Table 5: Clinical characteristics at baseline and change after 24 weeks.

Characteristics	At baseline, $n = 40$	After 24 weeks, $n = 40$	P value
Body mass (kg)	114.5 ± 26.4	108.1 ± 25.1	<0.001
BMI (kg/m^2)	40.9 ± 7.6	38.5 ± 7.1	<0.001
WC (cm)	122.3 ± 17.0	115.7 ± 16.2	<0.001
HbA1c (%)	8.4 ± 1.3	7.4 ± 1.3	<0.001
Glucose fasting (mmol/l)	8.9 ± 1.8	6.9 ± 1.5	0.004
SBP (mm Hg)	138.7 ± 14.7	129.6 ± 7.9	0.001
DBP (mm Hg)	84.5 ± 8.4	78.7 ± 4.9	0.001
TC (mmol/l)	5.2 ± 1.3	4.8 ± 1.1	0.215
HDL (mmol/l)	1.0 (0.8; 1.3)	1.1 (0.9; 1.3)	0.565
LDL (mmol/l)	3.1 ± 1.1	2.5 ± 1.2	0.189
TG (mmol/l)	1.9 (1.5; 3.2)	1.7 (1.4; 3.1)	0.076
C-peptide fasting (ng/ml)	3.5 ± 1.8	3.3 ± 2.1	0.569
C-peptide after test* (ng/ml)	5.0 ± 2.8	5.4 ± 2.9	0.066
HOMA-IR	7.2 ± 4.4	4.7 ± 2.7	0.089
HOMA-β	35.7 (24.5; 61.0)	35.3 (27.3; 57.3)	0.716

*2 hours after breakfast in meal tolerance test.

3.1.2. Hormone Levels. A comparison of the adiponectin, leptin, ghrelin, GIP, and GLP-1 levels before and after treatment was done. Data show a significant difference only in fasting GIP level and ghrelin after meal tolerance test level: GIP and ghrelin levels were higher after treatment (Table 6).

We have also conducted the assessment of hormone level changes depending on BMI and HbA1c reduction level. The results suggest that a greater elevation of the GIP level is observed in the "nonresponders" group (Tables 7 and 8).

3.1.3. The Results of the Completion of the Questionnaires. The results of the completion of the visual analogue scale to reveal attitude to the food before and after treatment display the significant rise in fullness sensation: 33.0 (17.5; 53.5) mm at baseline and 62.0 (41.5; 70.0) mm after 24 weeks of treatment (Figure 2).

The hunger, desire to eat, and perspective consumption senses were lower after treatment, but the decrease was not significant.

3.2. Predictors of Treatment with aGLP-1 Response

3.2.1. Body Weight Loss Predictors. An effective body weight reduction (≥5% from initial) was observed in 51.5% of the participants.

Statistical analysis revealed no significant association of sex, age, diabetes duration and initial BMI with body weight loss rate.

The comparison of hormone (involved in appetite regulation) levels with BMI reduction rate showed that ghrelin level 2 hours after breakfast in the meal tolerance test was lower in patients with body weight reduction ≥ 5%: 2.2 (1.3; 2.7) vs. 11.8 (4.8; 14.5), $P = 0.011$ (Figure 3):

In addition, in patients with body weight loss ≥ 5% fasting ghrelin and GLP-1 levels were higher (ghrelin 1.9 (1.5; 6.1) vs. 1.4 (0.3; 2.0) pg/ml; GLP-1 0.11 (0.02; 0.14) vs. 0.01 (0.01; 0.11) mmol/l), but the differences were not significant ($P = 0.061$ and 0.242, respectively). In Pearson rank correlation analysis with the percentage of BMI loss, the major correlation was with GLP-1 level, but there was also only weak correlation with a nonsignificant P value: $r = 0.384$ ($P = 0.064$). Percentage of BMI loss was calculated as $(\mathrm{BMI}_{\mathrm{baseline}} - \mathrm{BMI}_{\mathrm{after\,24\,months}})/\mathrm{BMI}_{\mathrm{baseline}} \times 100\%$.

Then, comparison of body weight loss rate taking into consideration appetite, hunger, fullness and type of eating behavior was performed. The only significant association was shown depending on the sensations of hunger: people with body weight loss < 5% have a stronger sense of hunger at baseline: 40.0 (25.0; 96.0) vs. 18.5 (10.0; 35.0), $P = 0.047$.

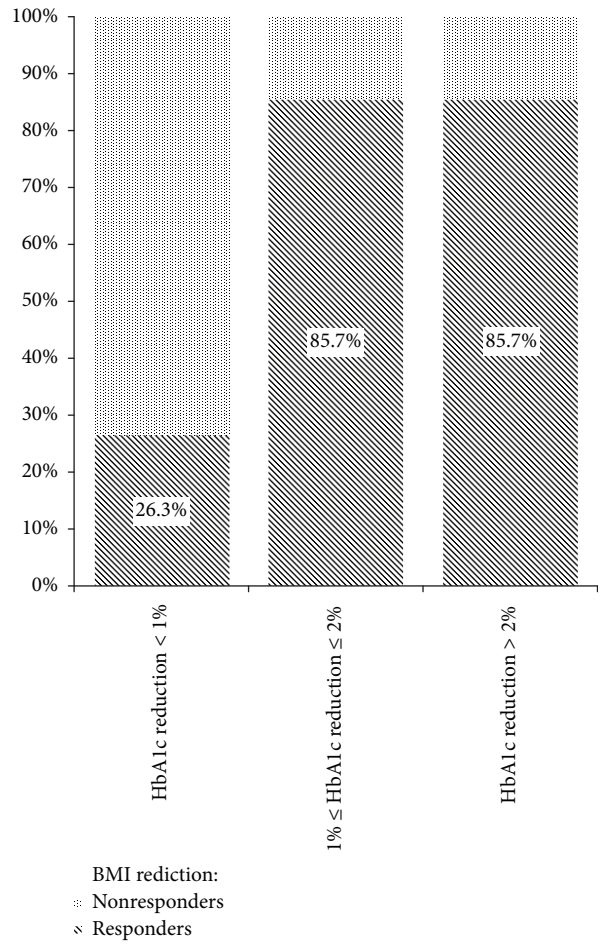


Figure 1: Percent of target BMI loss among groups with different HbA1c reduction levels.

The associations of body weight loss percentage with fullness, desire to eat, and perspective consumption sensations were not revealed.

The analysis of eating behavior types revealed no significant differences. Among patients with target weight loss, the percentage of those with signs of all three eating behavior types including restrained (restrained ± emotional ± external, in comparison with individuals with emotional ± external types) was higher than among patients with body weight reduction < 5%: 83.3% vs. 63.6%, but the difference was not significant ($P = 0.229$).

3.2.2. HbA1c Level Reduction Predictors. An effective HbA1c reduction (≥1%) was observed in 39.4% of the participants. Depending on the age, sex, and diabetes duration, the subgroups "responder" and "nonresponder" did not differ significantly. The statistical analysis showed that the HbA1c reduction rate was associated with HbA1c and GLP-1 initial levels. HbA1c reduced more intensively in patients with higher HbA1c and GLP-1 levels at baseline (Figures 4 and 5). In the Pearson rank correlation analysis, a moderate correlation was obtained between the HbA1c reduction rate and GLP-1 level with significant a P value: $r = 0.537$ ($P = 0.008$).

The sensations of appetite and fullness, as well as eating behavior types at baseline, were not associated with HbA1c reduction percentage.

3.2.3. Lipid Profile Improvement Predictors. Table 9 shows that a more effective decline of the TG level is associated with a higher initial TG and with lower GIP and ghrelin after test levels.

TG level decrease was also more prominent in the group with target weight loss (Table 10).

4. Discussion

As we did not find the differences between the mean reduction of BMI and HbA1c in patients taking liraglutide and exenatide, we combined these groups.

In our study, the proportion of patients who responded well to the treatment of aGLP-1 was similar to the results of other studies [7–12]. Meanwhile, we analyzed separately the response of different metabolic parameters (weight, glycemia, and dynamics of blood pressure and lipids) to the therapy and found that it may vary according to them. So, we revealed the decline in HbA1c by more than 1% only in 39.4% of the patients who reduced their weight by 5% or more. According to the literature, 30% of the patients lost ≥5% of body weight [35]. Also, in several other studies [17] a significant improvement in lipid parameters associated with visceral obesity was noted: a decline in the TG level, an increase in the HDL level and a fall in BP. We obtained a significant decrease in BP.

Our study demonstrated that aGLP-1 is more effective in obese patients with worse glycemic control. These results coincide with the literature. The most significant weight loss and HbA1c reduction in more obese patients and in the longest diabetes duration were shown. The longer duration of diabetes was noted to be a predictor of a greater weight loss in patients on exenatide [36]. Meanwhile, unlike these researchers [36], according to our study we did not show a correlation with diabetes duration, which may be due to a higher level of C-peptide (less pronounced disturbances in the functional reserve of β-cells) in our study.

In other research studies, the body weight loss due to the treatment with aGLP-1 (20 mg exenatide and 1.8 mg liraglutide per day) is more successful in patients with initially higher BMI [31, 36]. Our earlier results [16] confirmed it. However, in this study, we revealed only a trend toward better weight loss in patients with a higher BMI.

We, as well as other studies, demonstrated that the dynamics of blood pressure preceded the weight reduction [15], which indicates a weight-independent mechanism for improving this parameter. In addition, in our earlier study [16], as in some other studies [15], patients with a greater severity of hypertension demonstrated a more significant decrease in blood pressure.

The change in TG level naturally depended on the dynamics of weight and reached statistical significance only in patients who reduced their weight by 5% or more. Also,

Table 6: Hormones involved in appetite regulation levels at baseline and change after 24 weeks.

Hormone	At baseline	After 24 weeks	*P* value
GIP (pg/ml)	391.6 (326.6; 461.4)	429.9 (367.1; 504.0)	0.036
Ghrelin after test* (pg/ml)	2.7 (1.4; 3.5)	13.2 (2.1; 24.6)	0.041

*2 hours after breakfast in meal tolerance test.

Table 7: Glucose-dependent insulinotropic polypeptide level changes after 24 weeks of treatment in different groups depending on BMI reduction.

BMI reduction	At baseline	After 24 months	*P* value
Responders	418.7 (369.2; 488.8)	431.8 (392.4; 499.3)	0.289
Nonresponders	363.0 (241.5; 419.6)	412.1 (296.2; 478.3)	0.039

Table 8: Glucose-dependent insulinotropic polypeptide level after 24 weeks of treatment in different groups depending on HbA1c reduction.

HbA1c reduction	At baseline	After 24 months	*P* value
Responders	396.9 (358.7; 488.8)	429.0 (396.7; 469.3)	0.375
Nonresponders	376.0 (250.2; 436.7)	430.0 (338.1; 503.1)	0.039

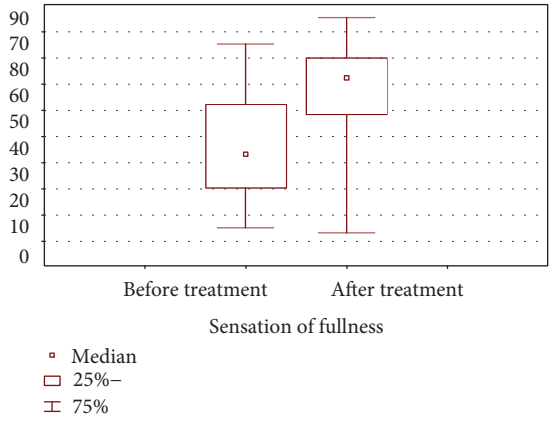


Figure 2: The sensations of fullness before and after treatment (VAS fasting state).

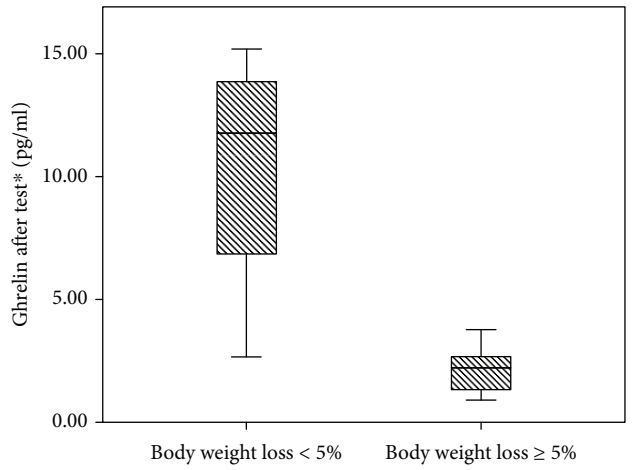


Figure 3: Ghrelin level after 2 hours in the meal tolerance test depending on body weight loss percentage.

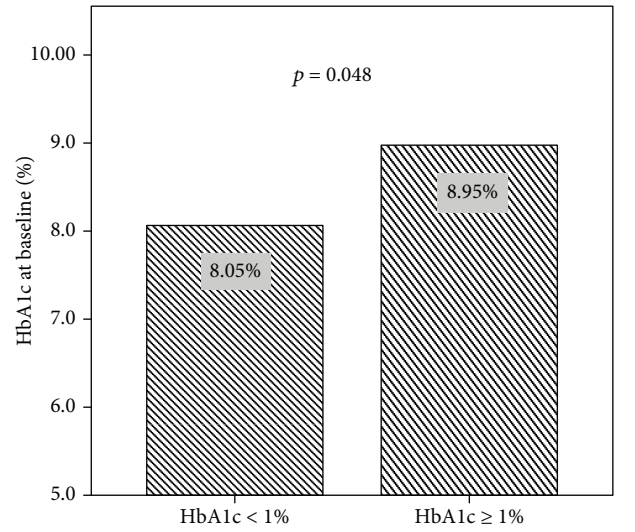
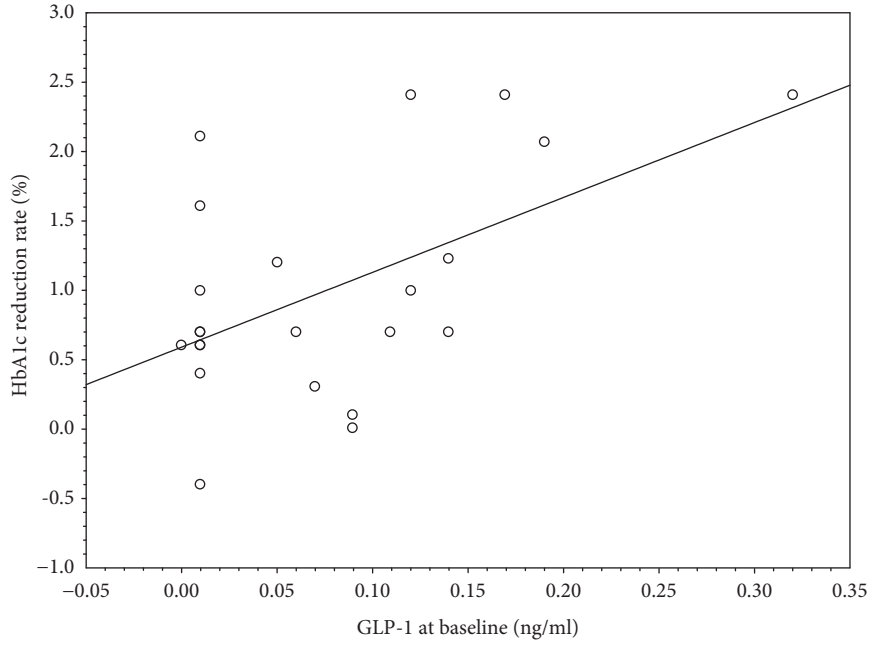


Figure 4: The association between the HbA1c reduction rate and HbA1c level at baseline (before aGLP-1 treatment).

the baseline level of TG was higher in those who reduced the TG level as a response to treatment by 30% or more.

There seems to be a feedback between the baseline levels and the obtained effect in almost all the studied metabolic parameters (weight, HbA1c, BP, and TG). Thus, the substrate-dependent nature of the response was noted for these metabolic parameters.

We also studied the level of hormones involved in the regulation of nutrient metabolism and appetite (GIP, GLP-1, leptin, adiponectin, and ghrelin). The basal levels of leptin and adiponectin were not shown as a predictor of the studied parameter dynamics in our research. Before treatment, we noted only a tendency to a slightly lower GIP level in weight nonresponders, but in the course of treatment the GIP level significantly increased in weight nonresponders. These differences seem logical, considering the role of GIP in the metabolism of adipose tissue (it is involved in fat intake from the gastrointestinal tract and their storage in white adipose tissue).



$r = 0.5371$; $p = 0.0082$

Figure 5: The association between the HbA1c reduction rate and GLP-1 initial level.

Table 9: Triglyceride level decrease predictors.

Factor (levels at baseline)	TG reduction ≥ 30%	TG reduction < 30%	*P* value
TG (mmol/l)	2.6 (2.2; 7.1)	1.6 (1.4; 2.0)	0.002
GIP (pg/ml)	331.5 (217.8; 368.5)	417.3 (380.3; 461.4)	0.019
Ghrelin after test* (pg/ml)	1.33 (0.88; 1.67)	2.65 (2.42; 11.79)	0.036

*2 hours after breakfast in the meal tolerance test.

Table 10: Triglyceride level decrease depending on body weight loss percentage.

TG (mmol/l)	At baseline	After 24 weeks	*P* value
Body weight loss ≥ 5%	1.97 (1.51; 3.53)	1.65 (1.23; 2.62)	0.02
Body weight loss < 5%	1.95 (1.43; 2.95)	1.8 (1.47; 2.68)	0.81

In our patients with T2DM and obesity, we found an abnormally low level of fasting ghrelin and no adequate reduction in the majority of the examined patients before treatment. Earlier, this fact was described by other authors in patients with obesity only and was explained as a manifestation of resistance to ghrelin. In the work of Crujeiras et al. [37], obese adults treated with a hypocaloric diet were studied. The authors noted that the low level of ghrelin before treatment and after 8 weeks of treatment was associated with an increased risk of weight regain (an increase in weight after an initial successful weight loss) in men. Our study showed that ghrelin was higher in fasting state and was lower in postnutritional status in patients with a body weight loss greater than 5%.

One more interesting finding of our study is a significant increase in ghrelin level in postnutritional status after treatment, in addition to the fact that the sense of satiety also increased significantly after 24 weeks of treatment with aGLP-1. Such dynamics of ghrelin level may indicate that patients who demonstrated good weight reduction in aGLP-1 are those who improved the sensitivity to ghrelin during the treatment. In addition, ghrelin is the regulator of GLP-1 secretion, and in the experiment, intraperitoneal injection of ghrelin to mice 15 minutes prior to oral glucose intake increased the glucose-stimulated secretion of GLP-1 and improved glucose tolerance [38]. On top of that, patients who reduced their weight by more than 5% had a higher fasting GLP-1 level. The results revealed that both the weight- and glucose-lowering effect of aGLP-1 in patients with a higher baseline level of GLP-1 indirectly confirms the results obtained in other studies about the importance of the preserved β-cell function for good efficacy of GLP-1 [12]. More severe GLP-1 deficiency would be expected to show the best results of aGLP-1 therapy due to the replenishment of deficiency. However, the current study showed the opposite results, which can be explained by the fact that a decrease in incretin secretion accompanies a decline in the β-cell reserve and even probably goes ahead of it, being a predictor of a poor response

to aGLP-1 therapy. In addition, the TG responders had lower baseline fasting levels of GIP and a trend toward a lower postprandial ghrelin level, which did not reach statistical significance.

A visual analogue scale and the Dutch food questionnaire can be a useful tool for weight loss prediction. The severe hunger according to VAS was associated with a poor weight reduction on aGLP-1 therapy. Patients who had elements of the restrictive type of eating behavior often had a notable weight loss. Identifying the dominance of the emotional component in the overeating genesis is the reason for involving the psychologist in weight correction in a patient with obesity.

The limitations of our study are the low number of cases, the mixed exenatide/liraglutide group of patients, and the absence of randomization as the study was conducted within the real medical practice.

Thus, we found that in patients on aGLP-1 therapy the predictors of glucose-lowering response are HbA1c and GLP-1 initial levels and the weight loss predictors are ghrelin level 2 hours after breakfast in the meal tolerance test and a less stronger sense of hunger at baseline. Predictors of an improving lipid profile are higher initial TG and lower GIP and ghrelin after meal tolerance test levels.

5. Conclusion

We found that the incretin level (GLP-1 initial level) predicts the glucose-lowering response on aGLP-1 therapy, the ghrelin level 2 hours after breakfast in the meal tolerance test predicts body weight reduction, and lower GIP and ghrelin after test levels predict TG level reduction. Identification of simple and accessible predictors of the response to aGLP-1 therapy will optimize the selection of patients for this therapy and improve not only glycemic control, but also the control of metabolic parameters which are cardiovascular risk factors (BMI, waist circumference, BP, and lipids).

Identification of patients who respond well to aGLP-1 therapy on the above parameters is expected to allow outlining a group of patients who will improve their cardiovascular prognosis on aGLP-1 therapy.

Abbreviations

aGLP-1: Glucagon-like peptide-1 receptor agonists
BMI: Body mass index
DBP: Diastolic blood pressure
SBP: Systolic blood pressure
GIP: Glucose-dependent insulinotropic polypeptide
GLP-1: Glucagon-like peptide type 1
HbA1c: Glycated hemoglobin
HDL: High-density lipoprotein
HOMA-IR: Insulin resistance index
HOMA-β: Index to estimate the functional reserve of β-cells
hs-CRP: High-sensitivity C-reactive protein
LDL: Low-density lipoprotein
T2DM: Diabetes mellitus type 2
TC: Total cholesterol
TG: Triglycerides
WC: Waist circumference.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

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

Acknowledgments

This study was carried out as part of the implementation of a scientific project supported by a grant from the Russian Science Foundation (Agreement No. 17-75-30052).

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Hindawi
Journal of Diabetes Research
Volume 2019, Article ID 2591709, 8 pages
https://doi.org/10.1155/2019/2591709

Research Article

Does Body Mass Index and Height Influence the Incident Risk of Ischemic Stroke in Newly Diagnosed Type 2 Diabetes Subjects?

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Received 28 June 2018; Revised 2 September 2018; Accepted 16 September 2018; Published 22 January 2019

Guest Editor: Markus Wallner

Objective. To estimate the incident risk of ischemic stroke (IS) in newly diagnosed type 2 diabetes (T2D) subjects according to different body mass index (BMI) and height categories. *Methods*. A total of 25,130 newly diagnosed T2D subjects were included in this study. All T2D subjects were enrolled consecutively from the Chronic Disease Surveillance System (CDSS) of Ningbo. Standardized incidence ratio (SIR) and its 95% confidence interval (95% CI) stratified by BMI categories and height quartiles were used to estimate the incident risk of IS in T2D subjects. *Results*. In total, 22,795 subjects completed the follow-up. Among them, 1268 newly diagnosed IS cases were identified, with 149,675 person-years. The SIRs of normal BMI (18.5–24.0 kg/m^2), overweight (24.0–28.0 kg/m^2), and obese (≥28.0 kg/m^2) in overall subjects were 2.56 (95% CI 1.90–3.13), 2.13 (95% CI 1.90–3.13), and 1.87 (95% CI 1.29–2.43), respectively ($P_{trend} < 0.01$), comparing to the general population of Ningbo. For each 1 kg/m^2 increment in BMI, the SIR was 0.948 (95% CI 0.903–0.999). For height quartiles, the SIRs of male subjects in quartile 1 (<160 cm), quartile 2 (161–165 cm), quartile 3 (165–170 cm), and quartile 4 (≥171 cm) were 2.27 (95% CI 1.99–2.56), 2.01 (95% CI 1.67–2.45), 1.37 (95% CI 1.05–1.68), and 0.91 (95% CI 0.40–1.32), respectively ($P_{trend} < 0.01$). While for female subjects, the SIRs in quartile 1 (<155 cm), quartile 2 (156–160 cm), quartile 3 (161–165 cm), and quartile 4 (≥166 cm) were 3.57 (95% CI 3.11–3.49), 2.96 (95% CI 2.61–3.31), 1.94 (95% CI 1.51–2.36), and 1.71 (95% CI 0.95–2.47), respectively ($P_{trend} < 0.01$). *Conclusion*. Compared to the general population of Ningbo, T2D subjects had a higher incident risk of IS. Furthermore, the IS incident risk was not only higher in newly diagnosed T2D subjects with normal BMI but also lower in taller newly diagnosed T2D subjects.

1. Introduction

Type 2 diabetes (T2D), a major public health burden, affects more than 370 million people around the world [1]. Accumulating evidence indicates that people with T2D have a threefold higher risk of stroke in some ethnicities [2–5]. Stroke, one of the leading causes of death in China, contributes to 1.6 million deaths annually [6–8]. Of which, about 80% of all patients suffer ischemic stroke (IS) [9].

Compelling evidence suggests a significant relationship between T2D and higher IS mortality [10]. Compared to overweight or obese subjects, those with normal weights were associated with higher IS mortality in both T2D subjects [11, 12] and the general population [4, 11–15]. Few studies have investigated the incident risk of IS in newly

diagnosed T2D subjects based on different categories of BMI [16], especially in Asians. And epidemiology studies revealed an inverse association between height and risk of stroke in adults [17–19]. A large meta-analysis involving 121 prospective studies reported that each 0.065 cm increase in height was associated with 6% (95% CI 3–10%) decreased risk of IS [20].

Taken together, we hypothesized that different BMI categories or height quartiles would experience different incident risk of IS in newly diagnosed T2D subjects. Therefore, the aim of this study was to estimate the incident risk of IS in newly diagnosed T2D subjects based on different categories of BMI and height quartiles with standardized incidence ratio (SIR).

2. Material and Methods

2.1. Study Population. Ningbo, a coastal city with a population of over seven million in 2015, is an economic center in Zhejiang Province. All the newly diagnosed T2D subjects included in this study were obtained from the Chronic Disease Surveillance System (CDSS) of Ningbo. The CDSS was established based on 11 monitoring sites, which are fully representative of the whole of Ningbo. The system was founded in 2002, with the aim to monitor the epidemic of chronic diseases (diabetes, cardiovascular disease, and cancer) and their risk factors. Residents who have lived in Ningbo for more than five years are included in the CDSS [21]. A total of 25,130 subjects at baseline were included in this study in accordance with the following inclusion criteria: newly diagnosed T2D subjects; diagnosed between January 1, 2006, and December 31, 2007; had available health records in the CDSS; and without myocardial infarction, heart failure, or stroke at baseline. All the included subjects were followed once per year, and loss to follow-up was defined as could not be contacted after three reasonable efforts. This study was approved by the institutional review board of Ningbo Municipal Center for Disease Prevention and Control. All the subjects provided written informed consent.

2.2. Outcome. All the newly diagnosed T2D subjects at baseline were connected to stroke diagnosis records (diagnosed between January 1, 2008, and December 31, 2014) in the CDSS, through each subject's full name, personal ID, and gender. IS was diagnosed according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST): a sudden onset of focal (or global) disturbance of cerebral function lasting > 24 h (unless interrupted by surgery or death) with no apparent nonvascular cause [22], with symptoms of large artery atherosclerosis, small artery occlusion, nonatherosclerotic vasculopathies, prothrombotic disorders, and cryptogenic cause [9, 22].

2.3. Demographic and Biochemical Measurements. Demographic and biochemical data were obtained from the medical records in the CDSS. Demographics included age, sex, weight, height, and education level. Biochemical measurements included fasting blood glucose (FBG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and glycated hemoglobin (HbA_{1c}). Blood glucose levels were measured by modified hexokinase enzymatic method. TC and HDL-C were analyzed enzymatically using commercial reagents, and LDL-C levels were calculated using the Friedewald equation. HbA_{1c} was measured by ion-exchange HPLC on a Bio-Rad Variant II instrument [23].

2.4. BMI and Height Classification. BMI was calculated in kg/m^2 according to each subject's height and weight. Weight was measured without shoes and in light clothing to the nearest 0.1 kg using a calibrated beam scale, and height of participants was measured without shoes to the nearest 0.2 cm using a portable stadiometer [24]. Subjects were divided into three groups: normal BMI group (18.5–24.0 kg/m^2), overweight group (24.0–28.0 kg/m^2), and obesity group (≥28.0 kg/m^2) [25], in accordance with the criteria issued by the National Health and Family Planning Commission of China. Sensitivity analysis by applying the World Health Organization criteria [normal weight (18.5<25 kg/m^2), overweight (25–29.9 kg/m^2), and obese (≥30 kg/m^2)] was also performed [26]. We also excluded the underweight subjects (BMI ≤ 18.5 kg/m^2), as lower body weight tended to be coexistent with obesity-related metabolic disorders, which were more susceptible to stroke [11]. For height, subjects were divided into quartiles: ≤160 cm, 161–165 cm, 166–170 cm, and ≥171 cm for males and ≤155 cm, 156–160 cm, 161–165 cm, and ≥166 cm for females, respectively.

2.5. Statistical Analysis. Continuous variables were presented as mean ± SD and categorical variables as absolute and relative frequencies (percentage). Baseline characteristics were summarized based on BMI categories and height quartiles. Comparisons of demographic and clinical variables between males and females were performed using t-tests and χ^2 tests, as appropriate. The number of person-years of follow-up was calculated from the baseline date to the diagnosis of outcomes, death, loss to follow-up, or December 31, 2014, whichever occurred first [27]. The primary outcome was incident IS.

Crude incidence rate (CIR) for IS was calculated by the number of new incidents of diagnosed IS divided by the number of observed person-years. SIR and its 95% confidence interval (CI) were calculated as the ratio of the observed to the expected number of newly diagnosed cancer cases with the Poisson regression model, in which sex (male or female) and education level (illiteracy, below college, or above college) were entered as categorical variable and factors such as age, FBG, TC, HDL-C, LDL-C, and triglyceride (TG) as continuous variables. The methodology followed the details of our published article [28]. Linear trends were tested by the Cochran-Armitage test for categorical variables and means test for continuous variables. P values less than 0.05 were considered statistically significant. All statistical analyses were conducted by SAS version 9.4 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Baseline Characteristics. A total of 22,795 subjects completed the follow-up, while 2335 subjects were lost to follow-up (Supplementary Table S1 and Supplementary Table S2). Baseline characteristics of the newly diagnosed T2D subjects stratified by BMI categories are shown in Table 1. Those who had normal BMI were older than those who were overweight and obese. Baseline characteristics stratified by height quartiles are presented in Table 2. Compared to taller counterparts, subjects who were shorter had higher FBG and HbA_{1c} levels in both males and females.

A total of 1268 newly diagnosed IS cases with 149,675 person-years were identified during the follow-up. The baseline characteristics of the IS cases are presented in Supplementary Table S3. For males, the average height and BMI were 168.23 ± 9.88 cm and 22.68 ± 5.83 kg/m^2, respectively; for females, the average height and BMI were 156.12 ± 10.54 cm and 23.01 ± 4.21 kg/m^2, respectively.

3.2. SIRs of IS among Incidents of T2D Subjects by Different Age Groups. SIRs of IS stratified by age are presented in Figure 1. Compared to the general population of Ningbo, SIRs increased with age until 60 years old. The 60+ age group had the highest risk for both males and females, with SIRs being 3.89 (95% CI 3.31–4.50) and 3.15 (95% CI 2.54–3.69), respectively, after adjusted for FBG, TC, HDL-C, LDL-C, TG, and education level. With the same adjustment, the overall SIRs for males and females were 1.41 (95% CI 1.29–1.53) and 1.45 (95% CI 1.32–1.57), respectively.

3.3. SIRs of IS among Incidents of T2D Subjects by Different BMI Categories. The overall SIRs in overall subjects were 2.56 (95% CI 1.90–3.13), 2.13 (95% CI 1.90–3.13), and 1.87 (95% CI 1.29–2.43) in normal BMI, overweight, and obese groups, respectively, after adjusted for age, FBG, TC, HDL-C, LDL-C, TG, and education level ($P_{trend} < 0.01$) (Figure 2). The SIR was 0.948 (95% CI 0.903–0.999) for each 1 kg/m^2 increment in BMI. Figure 2(a) shows that normal BMI subjects had higher SIRs than overweight and obese subjects for both males and females. With the same adjustment, the SIRs in males were 2.46 (95% CI 1.82–3.02), 2.01 (95% CI 1.43–2.59), and 1.76 (95% CI 1.08–2.45) in normal BMI, overweight, and obese groups ($P_{trend} < 0.01$), respectively. Each 1 kg/m^2 increment in BMI was associated with 8% lower risk (SIR 0.922, 95% CI 0.877–0.970) of IS. The SIRs in females were 2.67 (95% CI 2.11–3.14), 2.24 (95% CI 1.68–2.77), and 1.90 (95% CI 1.15–2.60) in normal BMI, overweight, and obese groups ($P_{trend} < 0.01$), respectively. Each 1 kg/m^2 increment in BMI was associated with 3% lower risk (0.977, 95% CI 0.933–1.022) of IS. Sensitivity analysis also showed similar results (Supplementary Figure S1).

3.4. SIRs of IS among Incidents of T2D Subjects by Different Height Quartiles. Figure 2 shows that each 1 cm increment in height had 7%, 8%, and 15% lower risk of IS in total subjects, males, and females, respectively. With the adjustment for age, FBG, TC, HDL-C, LDL-C, TG, and education level, the SIRs of IS stratified by different height quartiles are illustrated in Figure 2(b). For males, the SIRs for quartile 1 (≤160 cm), quartile 2 (161–165 cm), quartile 3 (166–170 cm), and quartile 4 (≥171 cm) were 2.27 (95% CI 1.99–2.56), 2.01 (95% CI 1.67–2.45), 1.37 (95% CI 1.05–1.68), and 0.91 (95% CI 0.40–1.32), respectively ($P_{trend} < 0.01$). For females, the SIRs were 3.57 (95% CI 3.14–4.01), 2.96 (95% CI 2.61–3.31), 1.94 (95% CI 1.51–2.36), and 1.71 (95% CI 0.95–2.47) for quartile 1 (≤155 cm), quartile 2 (156–160 cm), quartile 3 (161–165 cm), and quartile 4 (≥166 cm), respectively ($P_{trend} < 0.01$).

4. Discussion

This population-based prospective study suggested that newly diagnosed T2D had higher IS risk for all BMI categories and height quartiles. Compared to the subjects that were overweight and obese, newly diagnosed T2D subjects with normal BMI experienced higher risk of IS in the total, male, and female subjects.

Consistent with our study, Li et al. found that each 1 kg/m^2 increase in BMI was associated with 1.7% lower risk of IS among 29,554 incidents in Americans with T2D [16]. Eeg-Olofsson et al. also reported that a 5 kg/m^2 increase in BMI was related to increased risk of stroke among 13,087 incidents in T2D subjects [29]. In our study, subjects with normal BMI were older than the overweight and obese counterparts, and increased age is an established risk factor for stroke [30]. However, a recent study demonstrated that obesity was significantly associated with increased incident risk of cardiovascular (CVD) and mortality from CVD in 10 prospective cohort studies among North Americans [31]. And the China Kadoorie Biobank (CKB) study found that every 5 kg/m^2 higher BMI was associated with 30% increased risk of IS among over 0.5 million normal Chinese adults [32]. The discrepancy among different studies may be due to the differences in study design, sample size, duration of follow-up, and age at recruitment. Notably, the inverse association was attenuated in both males and females, and female T2D subjects had higher IS risk than male counterparts. The levels of LDL-C were higher in females than in males in our study, and females had congenitally smaller-caliber coronary arteries than males, both of which are associated with increased risk of IS [33, 34].

In our study, we observed an inversed association between increased height and IS risk in both males and females. Compared to subjects with shorter heights, subjects with taller heights were younger than their counterparts, and age is a well-known risk factor for IS [35]. In accordance with our findings, a Japanese cohort observed that taller subjects were younger than shorter subjects, and each 5 cm increase in height was significantly inversely related to stroke risk [18]. Other prospective cohort studies also observed similar associations [17, 36, 37]. Taller subjects had a lower risk of stroke due to stronger pulmonary function and thicker coronary vessel diameters, which reduced the risk of vessel occlusion, thus contributing to decreased risk of IS [38].

Socioeconomic factors, such as education [33], income [6], and wealth [39], are important confounding factors for height, because people in higher socioeconomic classes tend to be taller than people in lower socioeconomic classes.

Table 1: Baseline characteristics of T2D subjects according to different BMI categories.

Variables	18.5–24.0 kg/m^2			P	24.0–28.0 kg/m^2			P	≥28.0 kg/m^2			P
	Total	Males	Females		Total	Males	Females		Total	Males	Females	
Numbers (%)	12,318 (54.04%)	5913 (48.00%)	6405 (52.00%)	<0.001	8790 (38.56%)	4131 (46.91%)	4659 (53.09%)	<0.001	1687 (7.40%)	833 (49.38%)	854 (50.62%)	<0.001
Age (year)	65.71 ± 12.85	64.33 ± 11.91	65.86 ± 13.09	<0.001	64.03 ± 12.38	62.95 ± 12.47	64.94 ± 1183	<0.001	60.79 ± 14.10	58.42 ± 13.89	61.27 ± 15.10	<0.001
BMI (kg/m^2)	21.25 ± 3.16	21.77 ± 2.99	20.93 ± 3.05	<0.001	25.46 ± 1.10	26.88 ± 2.30	24.39 ± 1.76	<0.001	30.58 ± 8.38	32.46 ± 6.59	28.93 ± 8.16	<0.001
Education level (%)				<0.001				<0.001				<0.001
Illiteracy	4495 (36.49%)	1642 (36.53%)	2853 (63.47%)		1895 (21.59%)	863 (45.56%)	1032 (55.44%)		308 (18.26%)	132 (42.88%)	176 (57.12%)	
Below college	6608 (53.65%)	3475 (52.59%)	3133 (47.41%)		6227 (70.84%)	2891 (46.42%)	3336 (53.58%)		1219 (72.56%)	609 (49.95%)	610 (50.05%)	
Above college	1245 (9.86%)	796 (63.94%)	449 (36.06%)		668 (7.57%)	449 (67.22%)	219 (33.78%)		160 (9.18%)	92 (57.50%)	68 (42.50%)	
FBG (mmol/L)	9.19 ± 2.19	9.31 ± 1.67	9.04 ± 2.25	0.883	9.33 ± 2.99	9.42 ± 2.87	9.28 ± 3.31	0.562	9.76 ± 3.10	9.91 ± 3.48	9.65 ± 2.90	0.681
OGTT (mmol/L)	12.36 ± 3.96	13.24 ± 4.23	12.08 ± 3.77	0.322	12.67 ± 4.23	12.71 ± 3.94	12.59 ± 4.74	0.677	13.11 ± 4.03	13.37 ± 3.59	13.20 ± 2.99	0.203
TC (mmol/L)	4.76 ± 1.22	4.62 ± 1.17	4.84 ± 1.52	0.738	5.03 ± 1.59	4.95 ± 1.63	5.13 ± 1.44	0.481	6.02 ± 2.10	5.89 ± 2.47	6.28 ± 2.19	0.889
HDL-C (mmol/L)	1.41 ± 1.09	1.35 ± 1.17	1.46 ± 1.08	0.923	1.77 ± 1.27	1.74 ± 1.52	1.83 ± 1.19	0.717	1.15 ± 1.04	1.10 ± 1.02	1.22 ± 1.07	0.552
LDL-C (mmol/L)	2.82 ± 1.01	2.74 ± 1.37	2.88 ± 1.02	0.153	3.07 ± 1.56	2.95 ± 1.79	3.12 ± 1.56	0.244	3.21 ± 1.79	3.14 ± 1.86	3.27 ± 1.75	0.396
TG (mmol/L)	2.13 ± 1.89	2.12 ± 2.01	2.13 ± 1.87	<0.001	2.39 ± 1.77	2.35 ± 1.70	2.42 ± 1.29	<0.001	2.80 ± 2.41	2.76 ± 2.33	2.83 ± 1.92	<0.001
HbA_{1c} (%)	8.66 ± 2.51	8.81 ± 2.69	8.58 ± 2.11	<0.001	8.71 ± 2.13	8.89 ± 2.56	8.45 ± 2.03	<0.001	8.75 ± 3.08	9.11 ± 4.10	8.33 ± 3.65	<0.001

Data are presented as mean ± SD or number (percentage). FBG: fasting blood glucose; OGTT: oral glucose tolerance test; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglyceride; HbA_{1c}: glycosylated hemoglobin.

Table 2: Baseline characteristics of T2D subjects according to height quartiles.

Variables	Males				P	Females				P
	≤161 cm	161–165 cm	166–170 cm	≥171 cm		≤155 cm	156–160 cm	161–165 cm	≥166 cm	
Numbers (%)	1126 (11.78%)	1638 (17.13%)	3868 (40.45%)	2930 (30.64%)		3263 (24.66)	6085 (45.98%)	3093 (23.37%)	792 (5.99%)	
Age (year)	68.04 (13.57)	66.31 (12.22)	64.53 (12.50)	62.29 (12.37)	<0.001	68.79 (13.14)	65.13 (12.45)	62.37 (13.00)	61.45 (13.02)	<0.001
BMI (kg/m^2)	22.09 (7.64)	23.32 (3.37)	23.08 (3.07)	23.29 (3.14)	0.059	22.95 (7.09)	23.11 (3.13)	23.20 (3.30)	22.90 (3.46)	0.0601
Education level (%)					<0.001					<0.001
Illiteracy	266 (23.64%)	262 (16.00%)	610 (15.77%)	408 (13.92%)		1250 (38.31%)	1428 (23.47%)	744 (24.05%)	194 (24.49%)	
Below college	768 (68.21%)	1264 (77.17%)	2887 (74.64%)	2099 (71.64%)		1783 (54.64%)	4136 (67.97%)	2123 (68.46%)	543 (68.56%)	
Above college	92 (8.15%)	112 (6.83%)	371 (9.59%)	423 (14.44%)		230 (7.05%)	521 (8.56%)	226 (7.49%)	55 (6.95%)	
FBG (mmol/L)	9.93 (3.20)	9.88 (3.14)	9.82 (4.19)	9.77 (4.23)	0.004	9.32 (4.14)	9.21 (3.12)	9.00 (2.39)	8.98 (3.29)	0.002
OGTT (mmol/L)	14.62 (4.10)	14.33 (3.93)	13.92 (5.01)	13.79 (5.12)	0.018	12.06 (4.61)	12.02 (4.11)	11.96 (3.49)	11.90 (4.17)	0.019
TC (mmol/L)	5.11 (1.40)	5.04 (1.33)	4.93 (1.89)	4.88 (1.92)	0.351	5.29 (1.93)	5.27 (1.41)	5.23 (1.21)	5.19 (1.42)	0.360
HDL-C (mmol/L)	1.26 (0.79)	1.25 (0.66)	1.22 (0.90)	1.20 (1.05)	0.434	1.55 (1.43)	1.49 (1.26)	1.47 (0.87)	1.41 (1.27)	0.470
LDL-C (mmol/L)	2.91 (1.23)	2.87 (1.02)	2.79 (1.51)	2.77 (1.45)	0.381	3.09 (1.58)	3.00 (1.08)	2.98 (1.02)	2.93 (1.09)	0.384
TG (mmol/L)	2.49 (1.80)	2.44 (1.68)	2.30 (1.99)	2.21 (2.07)	0.395	2.49 (2.21)	2.42 (1.98)	2.40 (0.99)	2.39 (2.06)	0.402
HbA_{1c} (%)	8.75 (3.04)	8.72 (2.88)	8.62 (3.41)	8.63 (3.22)	0.301	8.33 (3.11)	8.27 (2.06)	8.26 (1.77)	8.21 (2.29)	0.311

Data are presented as mean (SD) or number (percentage). FBG: fasting blood glucose; OGTT: oral glucose tolerance test; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglyceride; HbA_{1c}: glycosylated hemoglobin.

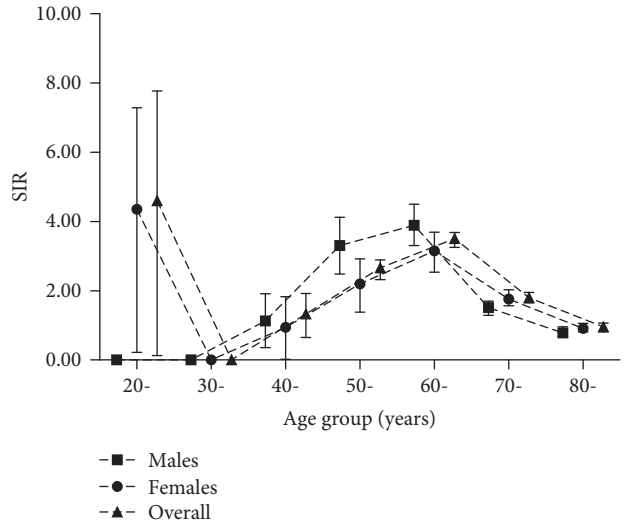

Figure 1: Standardized incidence ratios (SIRs) of ischemic stroke among incidents of type 2 diabetes subjects according to different age groups, adjusted for age, fasting blood glucose, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglyceride, and education level.

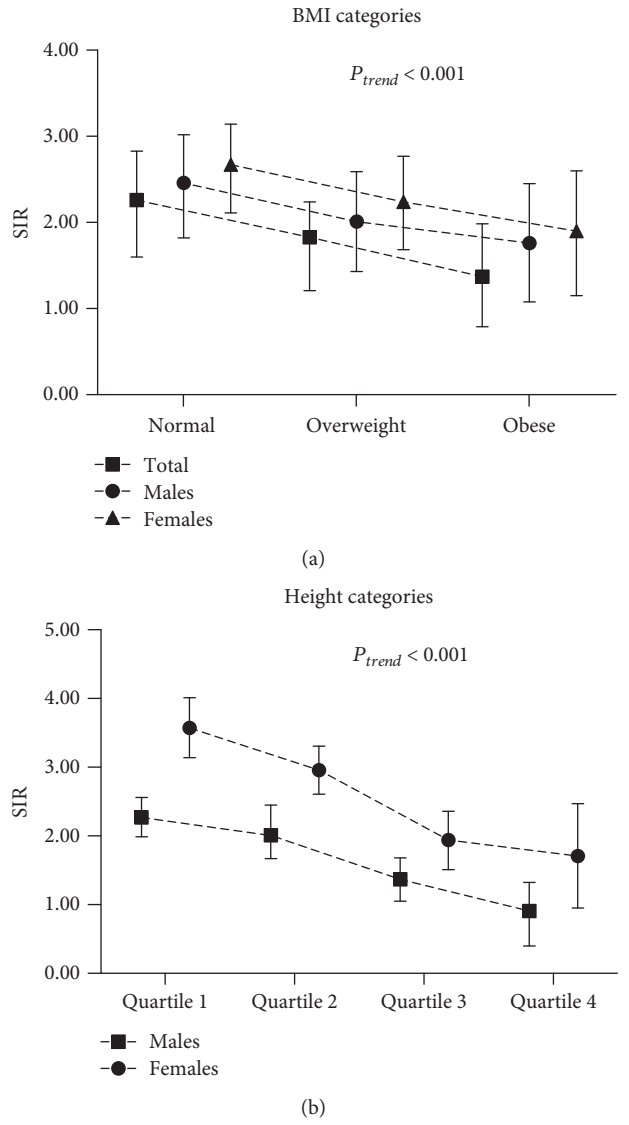

Figure 2: Standardized incidence ratios (SIRs) of ischemic stroke among incident type 2 diabetes subjects according to body mass index (BMI) categories and height quartiles, adjusted for age, fasting blood glucose, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglyceride, and education level.

Similar associations were reported in the association between BMI, education, and T2D [40]. Also, several biochemical indexes, such as FBG, LDL-C, and LDL-C, are also confounding factors for BMI or height, and most previous studies adjusted those indexes in the statistical models [16, 41, 42]. Therefore, we also adjusted those variables in our models.

To the best of our knowledge, we specifically estimated the incident of IS among newly diagnosed T2D subjects based on different BMI groups and height quartiles in the Chinese population. The larger sample size and longer duration of follow-up, and the inclusion of only newly diagnosed T2D subjects without preexisting cardiovascular disease at baseline, minimized the effect of T2D duration and preexisting diseases on IS risk. Moreover, we excluded subjects who were underweight, as lower BMI might indicate an underlying illness and susceptibility to IS [11].

Nevertheless, the following limitations should be acknowledged. Our data were obtained from the CDSS of Ningbo, and some important confounding factors were not available, such as smoking, alcohol consumption, and physical activity, which limited our further analysis. Besides, BMI was employed as the measure of adiposity, which does not reflect the overall fat distribution. Studies reported that waist circumference or waist-to-height ratio (WHR) might provide additional information beyond BMI for both incidence and mortality risk among middle-aged adults [43, 44]. And the generalizability of our findings is limited to newly diagnosed T2D subjects in Ningbo. Last, we could not stratify age into groups according to the life course (young, middle age, and older adulthood).

5. Conclusion

In conclusion, compared to the general population of Ningbo, T2D subjects had higher incident risk of IS. Newly diagnosed T2D subjects with normal BMI had a higher risk of IS compared to those who were overweight and obese, and increased height was related to decreased IS risk.

Data Availability

Previously reported data were used to support this study and are available at [doi:10.1016/j.canep.2018.02.006]. These prior studies (and datasets) are cited at relevant places within the text as references [28].

Disclosure

Donghui Duan, Hui Li, and Jiaying Xu are co-first author.

Conflicts of Interest

All authors have no competing financial interests or conflict of interests in the publication of this manuscript.

Acknowledgments

This study was funded by Sanming Project of Medicine in Shenzhen (SZSM201803080), National Key R&D Program of China (2017YFC1310902), National Natural Science Foundation of China (81402745), Natural Science Foundation of Zhejiang Province (LY17H260002), Natural Science Foundation of Ningbo (2016A610179), K. C. Wong Magna Fund of Ningbo University, Jiangsu Postdoctoral Research Foundation funded project (1601121B), National Natural Science Foundation of Ningbo (2017A610219), Ningbo Scientific Innovation Team for Environmental Hazardous Factor Control and Prevention (2016C51001), and Ningbo Municipal Medical and Health Brand Discipline "Field Epidemiology" (PPXK2018-10).

Supplementary Materials

Supplementary Table S1: baseline characteristics of 22,795 T2D subjects. Supplementary Table S2: baseline characteristics of the lost to follow-up subjects. Supplementary Table S3: baseline characteristics of 1268 T2D subjects with ischemic stroke. Supplementary Figure S1: SIR of IS among T2D subjects according to BMI categories. *(Supplementary Materials)*

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Hindawi
Journal of Diabetes Research
Volume 2018, Article ID 9242579, 9 pages
https://doi.org/10.1155/2018/9242579

Research Article

Early Subclinical Atherosclerosis in Gestational Diabetes: The Predictive Role of Routine Biomarkers and Nutrigenetic Variants

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Received 2 August 2018; Revised 17 October 2018; Accepted 30 October 2018; Published 24 December 2018

Academic Editor: Claudio De Lucia

Gestational diabetes mellitus (GDM) can be considered a silent risk for out-of-pregnancy diabetes mellitus (DM) and cardiovascular disease (CVD) later in life. We aimed to assess the predictive role of 3rd trimester lipid profile during pregnancy for the susceptibility to markers of subclinical atherosclerosis (CVD susceptibility) at 3 years in a cohort of women with history of GDM. A secondary aim is to evaluate the usefulness of novel nutrigenetic markers, in addition to traditional parameters, for predicting early subclinical atherosclerosis in such women in order to plan adequate early prevention interventions. We assessed 28 consecutive GDM women in whom we collected socio-demographic characteristics and clinical and anthropometric parameters at the 3rd trimester of pregnancy. In a single blood sample, from each patient, we assessed 9 single nucleotide polymorphisms (SNPs) from 9 genes related to nutrients and metabolism, which were genotyped by High Resolution Melting analysis. All women then attended a 3-year-postpartum follow-up and on that occasion performed an oral glucose tolerance test (OGTT, with 75 g oral glucose), the measurement of carotid artery intima-media thickness (cIMT), and analyses of metabolic parameters. In addition, we evaluated the physical activity level and the adherence to Mediterranean diet (MedDiet) using the International Physical Activity Questionnaire (IPAQ-*short version*) and PREDIMED questionnaires. We found an association between 3rd trimester triglycerides and cIMT ($p = 0.014$). We also found significant associations between the APOA5 CC genotype and cIMT after adjustments for age and body mass index ($p = 0.045$) and between the interaction CC APOA5/CC LDLR and cIMT ($p = 0.010$). At the follow-up, the cohort also featured a mean BMI in the overweight range and a high mean waist circumference. We found no difference in the MedDiet adherence, physical activity, and smoking but an inverse correlation between the PREDIMED and the IPAQ scores with the IMT. In conclusion, this preliminary study provides insight into the predictive role of lipid profile during pregnancy and of some genetic variants on cIMT taken as a parameter of subclinical CVD susceptibility in GDM.

1. Introduction

Gestational diabetes mellitus (GDM) is defined as "diabetes diagnosed in the 2nd or 3rd trimester of pregnancy, with or without remission after end of pregnancy" [1]. GDM prevalence has been reported to vary between 1% and 28% [2] and is increasing, especially in developed countries [3]. GDM may have clinical implications for both maternal and

fetal adverse outcomes and for the later development of type 2 diabetes in the years following pregnancy. In addition to type 2 diabetes, women with GDM are also at greater risk of overt cardiovascular disease (CVD) later in life. Several modifiable and unmodifiable risk factors are involved in the connection between GDM and subsequent CVD: these include hyperglycemia and impaired glucose tolerance, atherogenic lipid profiles, higher age, and elevated high-sensitivity C-reactive protein (CRP) [4]. Particularly, the development of diabetes purports an increased risk of developing later CVD [5, 6]. Nevertheless, mechanisms linking GDM and CVD are still unclear [6–11]. Metabolic impairments, including dyslipidemia and vascular dysfunction, are common later in life in women with previous GDM (pGDM) [12, 13]. In these women, elevated markers of inflammation, decreased levels of adiponectin, increased peripheral resistance, and decreased cardiac output have been detected [11]. Women with pGDM also have higher total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TG), as well as lower high-density lipoprotein cholesterol (HDL-C), compared with healthy women of the same age, suggesting that pGDM women have a greater susceptibility to be exposed to "an atherogenic insult" [14, 15]. Here, an obesogenic lifestyle (unhealthy diet and physical inactivity) and a genetic predisposition [16] likely explains the later development of CVD in most such women.

Carotid artery intima-media thickness (cIMT) is a subclinical measure of early atherosclerosis that strongly predicts heart disease and stroke, particularly in women [17, 18], and also predicts the development of CVD from GDM [4, 19].

Several studies recently carried out in women with pGDM have shown higher values of endothelial dysfunction markers and of cIMT in such women compared with controls, despite the absence of evident metabolic abnormalities [10, 19–21]. Previous studies of ours [22, 23] also showed a relationship between several nutrigenetic variants and cardiometabolic risk factors in women with or without GDM, suggesting the need to consider such factors in association with routinely assessed markers (such as lipid profile during pregnancy) for their role in the development of post-GDM CVD.

In this context, in the present study, we aimed to assess the joint predictive role of lipid profile during pregnancy and of some genetic variants on cIMT taken as a parameter of subclinical atherosclerosis and indicating an early susceptibility to CVD in a cohort of women with GDM history. If proven predictive, such prediction models would allow the planning of adequate primary cardiometabolic disease (CMD) prevention interventions in post-GDM women.

2. Materials and Methods

2.1. Study Design and Participants. Twenty-eight consecutive pGDM women attending the Diabetes and Metabolism Unit and the Obstetrics and Gynaecology Clinic, School of Medicine and Health Sciences, "G. d'Annunzio" University of Chieti-Hospital "SS Annunziata" of Chieti, were recruited. Socio-demographic characteristics and clinical parameters, such as blood glucose, TC, HDL-C, LDL-C, TG, and blood pressure, were collected. BMI was measured at the beginning (BMI 1) and at the end of pregnancy (BMI 2). A blood sample was obtained from each patient and nine single nucleotide polymorphisms (SNPs) from 9 genes related to nutrients and metabolism were included in the analysis.

All 28 women attended a 3-year-postpartum follow-up. Postpartum glucose tolerance (75 g oral glucose tolerance test (OGTT)) was assessed. Clinical parameters were collected in all subjects. Cardiovascular and metabolic markers were analyzed, including total, HDL, and LDL cholesterol levels; TG; homocysteine; and carotid artery IMT. In addition, HbA1C and fasting blood glucose were measured.

Adherence to the Mediterranean diet (MedDiet) was evaluated through a validated 14-item questionnaire (PREDIMED), which generates a range of possible scores namely (i) no adherence ($\text{score} \leq 5$), (ii) medium adherence ($6 \leq \text{score} \leq 9$), and (iii) maximum adherence ($\text{score} \geq 10$) [24]. In addition, physical activity (PA) was assessed using a *short version* of the International Physical Activity Questionnaire (IPAQ), comprising 7 items concerning PA, 4 relating to demographic information, and the remaining 6 about the comprehension of the questionnaire [25]. IPAQ registers three different levels of intensity (low, moderate, and high PA).

All participants gave their written informed consent prior to their inclusion in the study. The study was approved by the Ethics Committee of the "G. d'Annunzio" University, Chieti-Pescara, Italy.

2.2. Inclusion and Exclusion Criteria. The inclusion criteria admitted women with ≥18 years of age with pGDM. The GDM diagnosis was confirmed when established both at the 16–18th and the 24–28th weeks of gestation, according to the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria [26].

The exclusion criteria were women with type 1 or 2 prepregnancy diabetes, overt diabetes, or monogenic diabetes, specifically GCK diabetes.

2.3. Gene and SNP Selection. The genetic analysis was conducted at the Laboratory of Molecular Genetics, School of Medicine and Health Sciences, "G. d'Annunzio" University of Chieti. A total of 9 SNPs previously associated in literature with obesity, lipid, and glucose metabolism were selected. These SNPs, on different loci, have been associated with cardiovascular disease in previous studies, under the assumption that these variants may also contribute to cardiovascular risk assessment [22, 23].

In particular, three of these variants, namely, rs7903146 ($C > T$) in *TCF7L2*, rs1801282 ($C > G$) in *PPARG2*, and rs8192678 ($C > T$) in *PPARGC1A* are involved in carbohydrate metabolism; other three, namely, rs662799 ($T > C$) in *APOA5*, rs2228671 ($C > T$) in *LDLR*, and rs1260326 ($C > T$) in *GCKR* are involved in fat metabolism. In addition, two other SNPs, rs9939609 ($T > A$) in *FTO* and rs17782313 ($T > C$) near *MC4R*, associated with hunger control, were also selected. Finally, rs1801133 ($C > T$) in *MTHFR*, involved in folate metabolism, was also genotyped. It is of great relevance that all the selected gene variants make up a panel of CVD

Table 1: Characteristics of patients during pregnancy.

Variable	*N* = 28
Age (yr), *mean* ± *SD*	35.3 ± 3.8
School education, *n(%)*	
Low school	2 (7.1)
High school	14 (50.0)
University degree	12 (42.9)
Marital status, *n(%)*	
Single	5 (17.9)
Married	23 (82.1)
Employment, *n(%)*	
Employed	18 (64.3)
Unemployed	10 (35.7)
Prepregnancy BMI (kg/m^2), *mean* ± *SD*	27.4 ± 7.1
BMI at OGTT (kg/m^2), *mean* ± *SD*	30.8 ± 6.9
BMI at the end of pregnancy (kg/m^2), *mean* ± *SD*	31.6 ± 5.8
Weight variation, *mean* ± *SD*	
OGTT vs prepregnancy (kg)	6.2 ± 4.7
Delivery vs prepregnancy (kg)	6.3 ± 5.9
Systolic blood pressure (mmHg), *mean* ± *SD*	113.0 ± 16.8
Diastolic blood pressure (mmHg), *mean* ± *SD*	72.2 ± 10.9
3rd LDL-C (mg/dl), *mean* ± *SD*	150.5 ± 56.4
3rd HDL-C (mg/dl), *mean* ± *SD*	68.8 ± 13.5
3rd TC (mg/dl), *mean* ± *SD*	273.4 ± 51.0
3rd TG (mg/dl), *mean* ± *SD*	242.7 ± 75.8
Smoking habit, *n(%)*	
No	20 (71.4)
Yes	2 (7.1)
Ex	6 (21.4)
OGTT, *mean* ± *SD*	
T0	94.5 ± 6.3
T60	161.8 ± 27.1
T120	134.6 ± 34.5
Fasting blood glucose (mg/dl), *mean* ± *SD*	83.0 ± 8.0
Family history of DM (1st degree), *n(%)*	12 (42.9)
Previous GDM, *n(%)*	5 (17.9)

markers, which could provide a unique opportunity to use genetic information in clinical practice to predict early CVD in pGDM women [22, 23].

All SNPs were genotyped by High Resolution Melting (HRM) analysis. HRM was performed on 96-well PikoReal Real-Time PCR System (Thermo Scientific™) using the Luminaris Color HRM Master Mix (Thermo Scientific™) according to the manufacturer's instructions, as previously described [22].

2.4. Carotid Intima-Media Thickness Assessment. Carotid intima-media thickness (cIMT) was assessed in the Institute of Cardiology, School of Medicine and Health Sciences, "G. d'Annunzio" University of Chieti.

Left and right intima-media thickness (IMT) was measured on common carotid posterior wall, 1 centimeter from bulb bifurcation on each side, according to the latest European Society of Cardiology (ESC) and European Society of Hypertension (ESH) guidelines [27].

All the exams were performed with a dedicated Esaote MyLab 30 Gold portable ultrasound, with a standard 7.5 MHz linear probe, provided with Quality Intima Media Thickness (QIMT™) software. The software uses radio frequency data processing signal in real time and ensures high accuracy and low intra- and interobserver variability [28]. One expert operator (FB) performed all the exams in a blinded fashion from genetics; before the automatic measurement of QIMT, a manual image acquisition of carotid vessels was obtained with each participant lying supine, with the neck hyperextended. At each patient was attributed the mean value between three measurements for QIMT. Concordance correlation coefficient between the three measurements was 0.99 (95% CI: 0.98-0.99).

2.5. Statistical Analysis. We estimated the minimum required sample size for the correlation analysis on the basis of previously observed data or published results. The minimum sample size ($n = 28$) was determined in order to obtain an expected correlation coefficient ($r = 0.5$) [20] between cIMT and lipid profile parameters with at least 80% of desired statistical power level and an alpha error rate of 5%.

The quantitative variables were summarized as mean and standard deviation (SD) or median and interquartile range (IQR), according to their distribution. Qualitative variables were summarized as frequency and percentage. Shapiro-Wilk's test was performed to evaluate the departures from normality distribution for each variable.

Lin's concordance correlation coefficient (CCC) was calculated along with the 95% confidence intervals of assessing the intraobserver reproducibility of measurements.

Nonparametric Kruskal-Wallis test was performed to test the effect of different APOA5 genotypes on levels of cIMT and on levels of TC, HDL-C, LDL-C, and TG.

The relationship between cIMT and TC, HDL-C, LDL-C, and TG at the 3rd trimester was explored by linear multiple regression analysis adjusted for age and BMI. The univariate regressions between cIMT and lipid profile at the 3rd trimester of pregnancy were reported graphically as a scattergram.

Hardy-Weinberg equilibrium (HWE) deviations in the genotype frequency distributions were calculated using the chi-square analysis.

The level of statistical significance was set at $p < 0.05$. Statistical analysis was performed using the Statistical Package for Social Science (SPSS) software for Windows (SPSS, Chicago, IL, USA) and Stata v14.1 (StataCorp, College Station, TX).

3. Results

The demographic and clinical characteristics of the cohort of women, both during pregnancy and at follow-up, are reported in Tables 1 and 2, respectively. A total of 28 women were included in the study. No difference has been

Table 2: Characteristics of patients at follow-up.

Variable	$N = 28$
Age (yr), *mean ± SD*	37.9 ± 4.2
Height (m), *mean ± SD*	1.60 ± 0.06
Weight (kg), *mean ± SD*	71.4 ± 20.7
BMI (kg/m^2), *mean ± SD*	26.7 ± 9.2
Waist circumference (cm), *mean ± SD*	86.2 ± 16.1
Systolic blood pressure (mmHg), *mean ± SD*	118.5 ± 14.4
Diastolic blood pressure (mmHg), *mean ± SD*	74.8 ± 8.9
LDL-C (mg/dl), *mean ± SD*	110.1 ± 30.4
HDL-C (mg/dl), *mean ± SD*	54.2 ± 12.7
TC (mg/dl), *mean ± SD*	186.5 ± 33.2
TG (mg/dl), *mean ± SD*	110.8 ± 72.1
Fasting blood glucose (mg/dl), *mean ± SD*	91.4 ± 9.5
OGTT (mg/dl), *mean ± SD*	
T0	95.6 ± 9.4
T60	133.9 ± 35.7
T120	106.7 ± 24.4
HbA1C (mmol/mol), *mean ± SD*	35.4 ± 4.1
IPAQ, *n(%)*	
Low	14 (50.0)
Moderate	9 (32.1)
High	5 (17.9)
PREDIMED, *median* (Q_1-Q_3)	7.5 (6.0–9.0)
cIMT (mm), *mean ± SD*	0.51 ± 0.09
Homocisteina, *mean ± SD*	9.6 ± 3.1

found in the adherence to the MedDiet, PA, and smoking among the sample.

Although not statistically significant, an inverse correlation between the PREDIMED and the IPAQ scores with the cIMT values was found (Rho = −0.060, $p = 0.768$ and Rho = −0.276, $p = 0.163$, respectively). In addition, our results showed a connection between the women's waist circumference and cIMT values (Rho = 0.378, $p = 0.057$) at the follow-up, which, however, does not reach a statistical significance.

Furthermore, a significant positive relation between the 3rd trimester TG and cIMT (Rho = 0.468; $p = 0.014$) was found (Figure 1). The genotype distribution of investigated SNPs in patients is reported in Table 3. All the investigated genotype frequencies were within the Hardy-Weinberg ?equilibrium range (χ^2 test p value > 0.05). Also, a significant association was found in the codominant model (TT vs. TC vs. CC) between APOA5 CC genotype and cIMT after adjustments for age and BMI (0.50 ± 0.07 vs 0.48 ± 0.08 vs 0.65 ± 0.08; $p = 0.045$) (Figure 2). Finally, a significant association between the interaction CC APOA5/CC LDLR and cIMT ($p = 0.010$) has been observed.

No statistically significant differences were found among the 3rd trimester lipid profile, PREDIMED, and IPAQ scores in different APOA5 genotypes.

No other significant differences were detected with respect to other genes.

4. Discussion

The main aim of the present study was to assess the joint predictive role of lipid profile during pregnancy and of some genetic variants, cIMT taken as a parameter of subclinical atherosclerosis, and indicating an early susceptibility to CVD in a cohort of women with GDM history. Another aim was to examine new nutrigenetic markers as well as traditional parameters to predict early subclinical atherosclerosis in pGDM and to plan adequate early prevention interventions.

Several routine parameters and biochemical markers are currently available to quantify the risk in pGDM women to develop diabetes after pregnancy [3]. Although the incidence of CVD events in young women is low, an early identification of possible CMD risk may provide an irreplaceable opportunity for well-timed intervention and timely prevention. In order to reduce not only the occurrence of diabetes but also its subsequent cardiovascular complications, it is necessary to improve the provision of postpartum follow-ups. In fact, many authors have highlighted the close relationship between common CVD risk factors and a GDM history. In addition to traditional parameters, cIMT and arterial stiffness (RFQAS) values can be significant in assessing the risk for heart disease and strokes [18]. Bo et al. [20] measured cIMT in 82 women with a history of GDM and 113 without one, 6.5 years after delivery: their study showed that women with pGDM, regardless of their BMI and the presence of metabolic abnormalities, displayed remarkably higher E-selectin, ICAM-1, and IMT values than controls. IMT proved to be significantly associated with pGDM in a regression model, after adjustments for BMI, waist circumference, blood pressure, and glucose values. Volpe et al. [21] measured cIMT in 28 women with and 24 without a history of GDM 2 years after delivery, finding that young women with pGDM presented early signs of vessel involvement, albeit within upper normal levels. GDM and control groups differed in terms of their main metabolic syndrome components, such as waist circumference, blood pressure, fasting plasma glucose, and TG, all significantly higher in GDM women than in the control group.

Kaul et al. [29] observed that GDM was associated with 1.4 times higher rates of CVD. Then, Retnakaran and Shah [11] confirmed similar results, noting that women with GDM have an elevated risk of bad cardiovascular outcomes, even in the absence of type 2 diabetes.

Hypertriglyceridemia and low HDL-C are known to be characteristic traits of type 2 diabetes. It may not be surprising that in our previous studies their presence was detected in women with pGDM. Although the CV risk implications of hypertriglyceridemia and low HDL-C remain controversial, the CV significance of LDL-C and its main lipoprotein (apolipoprotein B (apoB)) is well established [30]. In our previous studies, GDM women showed significantly higher serum concentrations of TC and LDL-C during the 3rd trimester than the control group, and a significant correlation was

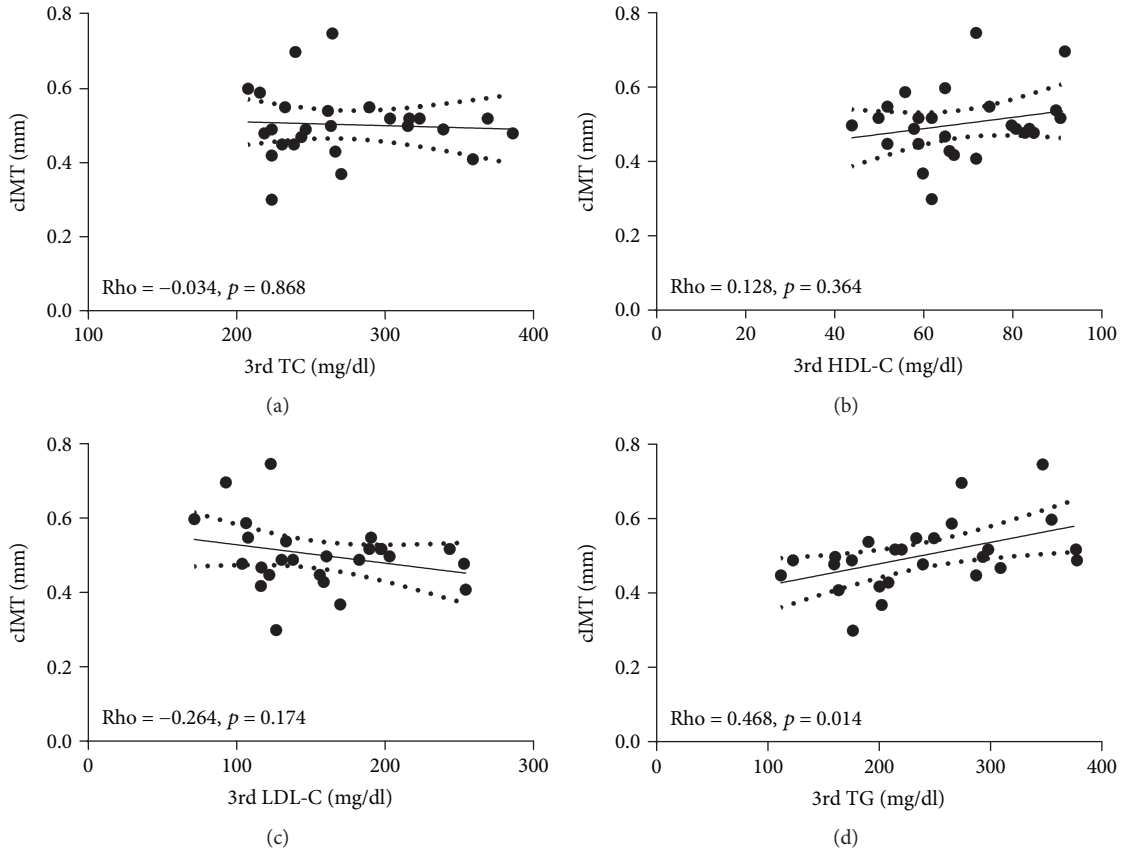

Figure 1: Nonparametric correlation analysis between cIMT and lipid profile during 3rd trimester of pregnancy.

Table 3: Genotypes distribution.

Genotype *n* (%)				No carrier	Carrier	HWE (*p* value)
TCF7L2	CC	CT	TT	CC	CT + TT	
rs7903146 (C > T)	8 (28.6)	10 (35.7)	10 (35.7)	8 (28.6)	20 (71.4)	*0.095*
PPARG2	CC	CG	GG	CC	CG + GG	
rs1801282 (C > G)	21 (75.0)	7 (25.0)	—	21 (75.0)	7 (25.0)	*0.389*
PPARGC1A	CC	CT	TT	CC	CT + TT	
rs8192678 (C > T)	9 (32.1)	16 (57.1)	3 (10.7)	9 (32.1)	19 (87.8)	*0.460*
APOA5	TT	CT	CC	TT	CT + CC	
rs662799 (T > C)	13 (46.4)	12 (42.9)	3 (10.7)	13 (46.4)	15 (53.6)	*0.857*
MC4R	TT	CT	CC	TT	CT + CC	
rs17782313 (T > C)	19 (67.9)	6 (21.4)	3 (10.7)	19 (67.9)	9 (32.1)	*0.099*
LDLR	CC	CT	TT	CC	CT + TT	
rs2228671 (C > T)	22 (78.6)	6 (21.4)	—	22 (78.6)	6 (21.4)	*0.460*
GCKR	CC	CT	TT	CC	CT + TT	
rs1260326 (C > T)	4 (14.3)	16 (57.1)	8 (28.6)	4 (14.3)	24 (85.7)	*0.293*
FTO	TT	TA	AA	TT	TA + AA	
rs9939609 (T > A)	8 (28.6)	10 (35.7)	10 (35.7)	8 (28.6)	20 (71.4)	*0.509*
MTHFR	CC	CT	TT	CC	CT + TT	
rs1801133 (C > T)	5 (17.9)	18 (64.3)	5 (17.9)	5 (17.9)	23 (82.1)	*0.190*

HWE = Hardy-Weinberg equilibrium.

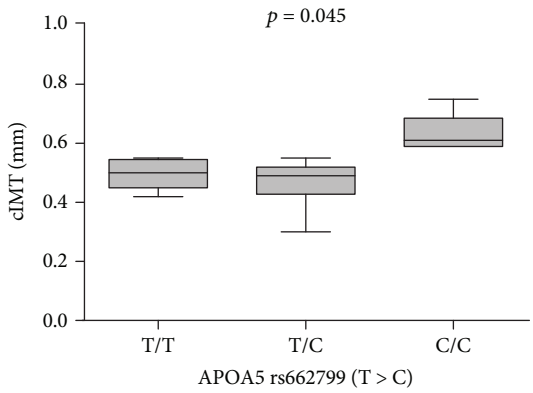

Figure 2: Box-whisker graphs of cIMT values with respect to APOA5 genotypes. Box-whisker plots show the 25th and 75th percentile range (box) with Tukey 95% confidence intervals (whiskers) and median values (transverse lines in the box). p value in figure is relative to Kruskal-Wallis test.

observed between lipid parameters and some polymorphisms in genes APOA5 and LDLR; also, TG were higher in GDM women than controls, although not reaching statistical significance [23]. Interestingly, in our present study, an association between 3rd trimester TG and cIMT has been found ($p = 0.014$).

These remarkable results echo those of Di Cianni et al. [31], who reported that TC, TG, LDL-C, glucose, and systolic blood pressure were all significantly higher among the GDM cohort, suggesting a condition similar to the metabolic syndrome occurring in these women. These peculiar changes in lipoprotein profile may favour endothelial damage in pregnancy [32, 33]. The GDM lipid profile is very similar to the one accompanying the insulin resistance in the metabolic syndrome. More recently, Gongora and Wenger [34] showed that women with GDM have a more atherogenic lipid profile by three months postpartum, characterized by an increase of cIMT compared to controls and that the risk of developing metabolic syndrome increased by up to 10% in those with pGDM.

It is known that pregnancy is a stress test, as hyperglycemia seems to have a significant impact on the CV system during this limited period [35]; we could hypothesize that lipid pattern modifications during pregnancy with GDM are an injury which results in a possible susceptibility to future CVD risk. Unfortunately, the mechanisms involved in an increased risk of CVD in pGDM need to be further investigated. GDM women's lipid profile displays a preponderance of small dense LDL particles; also, they present an increased susceptibility of LDL oxidation during pregnancy [30].

These data reveal the necessity of monitoring women with GDM adequately and long term, to prevent both CVD and diabetes risks. Unfortunately, several studies showed low rates of postdelivery glucose testing [36, 37]. It should be emphasised that, to date, the postpartum screening of women with pGDM is still suboptimal [3]; therefore, this issue also prompts the need to identify a practical and feasible tool which ideally should include panel genes and routine clinical and metabolic parameters, to identify GDM women at high risk of diabetes and CVD [23] and summon them for follow-ups. Previous investigations highlighted that women with pGDM have an increased risk of CVD later in life as a result of a combination of genetic factors and gene-diet interaction.

Furthermore, it is essential to define the characteristics of the studied gene variants. Interestingly, a previous Italian study showed that APOA5-1131T > C may affect the risk of early-onset myocardial infarction (MI), with an odds ratio of 1.44 (CI: 1.23–1.69) per C allele [38]. Apolipoprotein A-V gene, as described for the first time in 2001, is located proximal to the APOAI, APOCIII, and APOA-IV gene cluster on human 11q23 [39]. APOA5 encodes apolipoprotein (apo) AV, which is expressed in the liver and circulates on chylomicrons (CM), very low density lipoproteins (VLDL), and HDL. Common genetic variants of the apolipoprotein gene family members are related to variations in serum lipid levels.

Recently, a meta-analysis [40] showed that the APOA5 rs662799 C allele is associated with elevated circulating TG levels, regardless of ethnicity, indicating a possible mediating role for circulating TG in the association between the risk variant at APOA5 and the atherosclerotic process [41, 42]. This variant was correlated with not only higher plasma TG but also with lower HDL-C levels by our [22, 23] and other groups [38, 43]. Emerging data about APOA5-1131T > C suggested that APOA5 gene may have a direct effect above and beyond its effect on TG but, until now, needs to be confirmed by further studies [38]. In the Framingham Heart Study, an almost 2-fold increased risk of CVD was observed in females carrying the C allele of the −1131T > C [44]. Moreover, the relationship with IMT was observed for the rare allele of the −1131T > C SNP in overweight and obese subjects [45, 46].

Qiao et al. [47] observed that in type 2 diabetes patients, TG levels and the TG/HDL-C ratio were greater in those with TC and CC genotypes than in those with TT genotype subjects ($p < 0.05$). In addition, diabetic patients with CC genotype had greater carotid IMT than those with TT genotype ($p = 0.080$), although these data do not reach statistical significance.

We found a significant association between APOA5 CC genotype and cIMT ($p = 0.045$). Current evidence indicates that the increased risk of CVD is influenced by a merging of modifiable risk factors, ages, and/or genetics; however, the proportion of the contribution of these factors in modulating is still debated. Our findings suggest that even though the age of our study cohort was very young, women with C genotype probably experience the disadvantage of such genetic factor leading to CVD susceptibility in the form of a cIMT increase.

LDLR gene can regulate cholesterol metabolism. Among the several genetic variants identified at LDLR locus, the rs2228671 has been intensively studied and it has shown the strongest association with total and LDL-C levels across multiple populations [48–50] with the T allele being associated consistently, to a decreased risk of CAD [48]. Our previous findings are consistent with the previous literature; in fact, we showed that

carriers of the rs2228671 T allele were significantly associated with the 3rd trimester LDL-C levels in GDM women [22, 23].

Furthermore, we observed a significant association between CC APOA5/CC LDLR interaction and cIMT ($p = 0.010$). Surprisingly, women with CC genotype in APOA5 rs662799 in the absence of the T protective allele of LDLR rs2228671 present a cIMT increase. It would be interesting to further investigate the causal molecular mechanism underlying this interaction. We could hypothesize that the interaction effect of LDLR rs2228671 and APOA5 rs662799 implies a probable mitigation on cIMT values.

As expressed by Mecacci et al. [35], GDM can be considered as a "window into future health" in which it is advisable to adopt a healthy lifestyle to prevent or delay diabetes and/or CVD development postpartum.

In our previous study [23], we found that women with GDM had a greater BMI than the control group both in prepregnancy and at the end of pregnancy. In the present study, we analyzed women's lifestyle at follow-up: the pGDM women had a mean BMI that falls within the overweight range, as well as a high mean waist circumference (86.2 ± 16.1 cm). Moreover, no difference of adherence to the MedDiet, PA, and smoking was observed, although an inverse correlation between both the PREDIMED and the IPAQ scores with the cIMT values was found. This is an interesting feature supporting the potential role of preventive intervention. In fact, we noted that our cohort had a median MedDiet score of 7.5 (medium adherence), suggesting a poor adherence to healthy nutritional habits; also, a high percentage of them reported lower levels of PA. These modifiable risk factors can be easily acted upon to allow for early cardiovascular prevention.

In this view, tailored nutrition and lifestyle prescription represent a promising strategy for the prevention and management of metabolic syndrome [51]. In this regard, the main goals are a correct identification and stratification of GDM women at risk for CVD and an evaluation of the preventive strategies, as well as the improvement of postpartum screening.

Longer-term studies are indicated to define a potential role of lifestyle intervention [4]. Follow-up after GDM could be enhanced by similar quality and accountability measures requiring that patients and clinicians discuss future risks and referral to primary care as a standard of practice [36].

To our knowledge, this is the first study using multisectoral innovative biomarkers to evaluate the cardiometabolic risk in pGDM. Therefore, women with pGDM could be enrolled in follow-up programs designed to ensure continuous monitoring, thus providing effective prevention of both type 2 diabetes and CVD [7, 15].

It would be clinically valuable to have a risk marker during pregnancy so that long-term follow-ups and appropriate strategies of interventions can be focused on the women at greatest risk in a timely manner [52].

This study has some limitations. First of all, the sample size: this may have limited the statistical significance of metabolic and vascular function data. Second, non-pGDM women have not been involved. The present research is a preliminary small-scale study to evaluate the joint predictive role of lipid profile during pregnancy and of some genetic variants on cIMT taken as a parameter of subclinical atherosclerosis in a cohort of women with GDM history. Results obtained about the potential role of routine biomarkers and nutrigenetic variants in our small sample could be validated in a larger study.

However, our study provides a remarkable insight into the potential predictive role of both genetic factors and 3rd trimester lipid profile for CVD susceptibility in pGDM. It will be crucial to replicate and expand our findings and further studies are warranted for better understanding of the potential gene-gene and gene-environment interactions, thus attributing a significant prognostic role to new and old biomarkers during pregnancy.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interests associated with this manuscript.

Authors' Contributions

The study was designed by EV, LS, RDC, and MF. CC and ML contributed to clinical evaluation and support to the recruitment of patients. MF, FF, MDN, and EV contributed to data acquisition, analysis, and interpretation. The experiments were performed by MF. Nutritional status was assessed by FF. Carotid intima-media thickness was assessed by FB. The manuscript was drafted by MF, FF, and EV. DM contributed to data analysis. MDN was the statistician that performed the statistical analyses; she also helped in the drafting and editing of the manuscript. All authors were involved in critical revision and approved the final version of the manuscript before submission. EV, LS, and MDN are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Marica Franzago and Federica Fraticelli contributed equally to this work.

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Hindawi
Journal of Diabetes Research
Volume 2018, Article ID 4028297, 12 pages
https://doi.org/10.1155/2018/4028297

Research Article

L-Carnitine: An Antioxidant Remedy for the Survival of Cardiomyocytes under Hyperglycemic Condition

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Received 19 July 2018; Revised 13 September 2018; Accepted 11 October 2018; Published 9 December 2018

Guest Editor: Gaetano Santulli

Background. Metabolic alterations as hyperglycemia and inflammation induce myocardial molecular events enhancing oxidative stress and mitochondrial dysfunction. Those alterations are responsible for a progressive loss of cardiomyocytes, cardiac stem cells, and consequent cardiovascular complications. Currently, there are no effective pharmacological measures to protect the heart from these metabolic modifications, and the development of new therapeutic approaches, focused on improvement of the oxidative stress condition, is pivotal. The protective effects of levocarnitine (LC) in patients with ischemic heart disease are related to the attenuation of oxidative stress, but LC mechanisms have yet to be fully understood. *Objective*. The aim of this work was to investigate LC's role in oxidative stress condition, on ROS production and mitochondrial detoxifying function in H9c2 rat cardiomyocytes during hyperglycemia. *Methods*. H9c2 cells in the hyperglycemic state (25 mmol/L glucose) were exposed to 0.5 or 5 mM LC for 48 and 72 h: LC effects on signaling pathways involved in oxidative stress condition were studied by Western blot and immunofluorescence analysis. To evaluate ROS production, H9c2 cells were exposed to H_2O_2 after LC pretreatment. *Results*. Our *in vitro* study indicates how LC supplementation might protect cardiomyocytes from oxidative stress-related damage, preventing ROS formation and activating antioxidant signaling pathways in hyperglycemic conditions. In particular, LC promotes STAT3 activation and significantly increases the expression of antioxidant protein SOD2. Hyperglycemic cardiac cells are characterized by impairment in mitochondrial dysfunction and the CaMKII signal: LC promotes CaMKII expression and activation and enhancement of AMPK protein synthesis. Our results suggest that LC might ameliorate metabolic aspects of hyperglycemic cardiac cells. Finally, LC doses herein used did not modify H9c2 growth rate and viability. *Conclusions*. Our novel study demonstrates that LC improves the microenvironment damaged by oxidative stress (induced by hyperglycemia), thus proposing this nutraceutical compound as an adjuvant in diabetic cardiac regenerative medicine.

1. Introduction

Cardiovascular complications are recognized as the primary cause of mortality in subjects with diabetes mellitus (DM) [1, 2], characterized by hyperglycemia which is determined by a defect of insulin secretion, insulin action, or both [3]. Moreover, DM is associated with inflammation condition.

Chronic hyperglycemia, characterizing overt diabetes, or fluctuant hyperglycemia, present in the prediabetic condition, are responsible for the activation of numerous signaling pathways that exacerbate the systemic inflammation and lead to the development of diabetic complications [4, 5]. Recent evidence establishes how hyperglycemia is involved in the regulation of sirtuin (SIRT) transcription factors. The deregulation of SIRT expression is strictly correlated with the progression of inflammation and atherosclerotic disease. In particular, Balestrieri et al. demonstrated that SIRT6 protein expression is downregulated in atherosclerotic plaques of diabetics, and this defect is linked to the chronic oxidative stress condition [6]. Those evidences indicate that chronic

inflammation, oxidative stress condition and predisposition to ischemic heart disease are higher in patients with DM than in nondiabetics [7, 8].

Traditional therapeutic approaches as well as innovative promising strategies [7] (i.e., stem/progenitor cell therapy, existing cardiomyocyte proliferation, and reprogramming noncardiac cells) are limited in patients with DM [8–11]. Periprocedural intensive glycemic control, during early percutaneous coronary intervention in diabetic patients, was shown to improve myocardial protection by increasing SIRT1 expression, endothelial progenitor cell number, and their capability to differentiate in mature cardiomyocytes [12]. Hyperglycemia in diabetic subjects is the major factor responsible for the failure of regenerative myocardial therapeutic strategies. Recent data indicate that the overproduction of reactive oxygen species (ROS) and the oxidative stress condition are the main causes involved in diabetic cardiac injury and in the lack of success in cardiac regenerative therapies [13–17]. Hyperglycemia enhances ROS production impairing cardiac microenvironment and regeneration capacity [16, 17]. In particular, several publications showed that the hyperglycemic and oxidative microenvironment induces mitochondrial abnormalities and cellular damage, eventually leading to senescence and apoptosis of cardiac progenitors [18, 19].

Thus, to optimize regenerative strategies for diabetic patients, the development of new therapeutic approaches focused on the reduction of oxidative stress condition is fundamental.

Noteworthy, in recent years, L-carnitine (LC) has been proposed as a nutraceutical integrator in the treatment of numerous cardiac syndromes, including coronary disease, atherosclerosis, and toxic myocardial injury [20–22]. It is well established that LC, facilitating transport of long-chain fatty acids into the mitochondrial matrix, plays an important role in supporting cardiac energy homeostasis [23–25]. Most importantly, some studies successfully showed LC's ability to reduce oxidative stress, hypoxic cellular damage, and apoptosis of cardiac cells in normal glycemic condition [26, 27]. In particular, Mao et al. have recently demonstrated that LC pretreatment ameliorated cellular damage induced by H_2O_2 in H9c2 rat cardiomyocytes, enhancing mitochondrial function [28].

LC effects on cardiac metabolism and function have been demonstrated under a variety of clinical conditions [29, 30], while LC antioxidative effects in hyperglycemic cardiomyocites were not investigated, yet.

The aim of this study is to investigate *in vitro* LC ability to counteract oxidative stress condition, modifying ROS production and promoting mitochondrial detoxifying function in H9c2 rat cardiomyocytes under hyperglycemic condition (25 mmol/L glucose). The goal is to identify in LC a novel adjuvant agent in cell therapy able to ameliorate the microenvironment of the hyperglycemic heart, thereby supporting cardiac progenitor cell proliferation and differentiation.

2. Materials and Methods

2.1. Chemicals and Reagents. All utilized reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

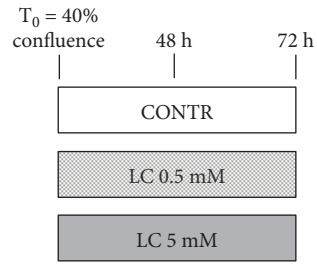

Figure 1: Scheme of treatment. Upon reaching 40% confluence, H9c2 cells were treated for 48 and 72 h with 0.5 or 5 mM LC.

Primary antibodies against GAPDH (FL-335), p-CaMKII α (22B1), CaMKII (M-176), AMPK α1/2 (H-300), SOD2 (FL-222), p21 (C-19), peroxidase-conjugated secondary antibodies for Western blot analysis, and FITC-conjugated antibodies for immunofluorescence study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). STAT3 (124H6) and p-STAT3 (Ser727) were purchased from Cell Signaling Technology (Danvers, MA, USA).

The CellROX® Oxidative Stress Reagent kit (C10443) was purchased from Thermo Fisher Scientific, Life Technologies Italia (Monza, Italy).

2.2. Cell Line and Culture Conditions. The American Type Culture Collection (ATCC, Manassas, VA, USA) offered the cardiomyoblast H9c2 cell line of rat embryo. The H9c2 cell line was regularly cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 25 mmol/L glucose, 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin, and 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) in 75 cm^2 tissue culture flasks and then incubated at 37°C by adopting 5% CO_2. H9c2 cells exposed to high glucose (25 mmol/L) represent a validated *in vitro* model in order to mimic cardiac hyperglycemic/diabetic condition [31, 32]. Cells were fed every day; following literature indications, H9c2 cells were split when reaching 70–80% confluence in order to prevent the loss of the differentiation potential [28, 32]. Preliminarily, we evaluated the effective concentration of LC based on our previous work performed on skeletal myoblasts and literature evidence (data not shown) [28, 33]. We identified the correct LC concentrations as the lower concentrations that activated STAT3 signaling. Then, H9c2 cells in the active proliferation phase were treated with 0.5 or 5 mM LC as indicated in Figure 1.

2.3. Growth Curve and Cell Viability Test. H9c2 cells were plated on 60 mm × 15 mm culture dishes at 20% confluence and grown in DMEM. After an overnight phase, the cells were treated or not with 0.5 or 5 mM LC. At 24, 48, and 72 h after treatments, cells were trypsinized and stained with trypan blue and were counted using hemocytometer. The average values for each single day were used to plot a growth curve. Cell viability was calculated by dividing the unstained viable cell count by the total cell count. In addition, morphological changes were observed daily by phase contrast microscopy.

2.4. Western Blot Analysis. Western blot analysis was performed as described previously [34]. Cell lysates were prepared using RIPA buffer implemented with protease inhibitors. 30 μg of proteins was separated by SDS-polyacrylamide gel electrophoreses (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes (Protran®, Whatman® Schleicher & Schuell). The blots were then blocked and incubated with specific primary antibodies, followed by incubation with anti-species-specific secondary antibodies. To confirm equal protein loading per sample, we used GAPDH protein as housekeeping protein. Finally, detection of specific proteins was performed by enhanced chemoluminescence reagent (Western Lightning ECL Pro, Perkin Elmer). Quantitative measurement of immunoreactive band intensities was performed by densitometric analysis using the Scion Image software (Scion Corporation, Frederick, MD, USA). Data were then converted into fold changes (FC) of the control.

2.5. Immunofluorescence Studies. H9c2 cells were grown on coverslips with or without LC 0.5 or 5 mM LC. After 48 and 72 h of treatment, cells were washed 3 times with PBS, then fixed in prepared 4% paraformaldehyde for 20 minutes. The cells on the coverslips were washed with PBS and incubated for 30 minutes at room temperature with 1% bovine serum albumin in PBS with 0.2% Triton X-100. At that point, H9c2 cells were incubated with primary antibodies for 120 minutes. To detect the primary antibody, binding site cells were washed three times in PBS and followed by incubation with specific antibodies FITC-conjugated for 90 minutes. Nuclei were revealed with DAPI staining. Coverslips with cells were mounted and observed using Nikon Eclipse 50I microscopy. The images were captured using Nis-Elements D 4.00 software. Immunofluorescence signals were estimated using the ImageJ program (http://imagej.nih.gov/ij/). Data were displayed and analyzed using Adobe Photoshop CS4®.

Automated quantification on the immunofluorescence signal was performed by using the ImageJ program (http://imagej.nih.gov/ij/) [34]. For each analyzed protein, the quantified signal was normalized for the total nuclei number.

2.6. Intracellular ROS Determination in H9c2 Cells after H_2O_2 Injury. Intracellular ROS levels were valued using the CellROX® Oxidative Stress Reagent kit. Briefly, H9c2 cells were pretreated with 0.5 or 5 mM LC for 48 h, and then they were exposed to 500 μM H_2O_2 for 30 min. At the end of the H_20_2 injury, the fluorogenic probe of the kit was added (Figure 2(a)). CellROX® Oxidative Stress Reagents are fluorogenic probes designed to reliably measure ROS in live cells. The cell-permeable reagents are nonfluorescent or very faintly fluorescent while in a reduced state and during oxidation exhibit a strong fluorogenic signal. The CellROX® Orange Reagent signal is localized in the cytoplasm.

The images were captured using Nis-Elements D 4.00 software. Data were displayed and analyzed using Adobe Photoshop CS4®.

Automated quantification on the immunofluorescence signal was performed by using the ImageJ program (http://imagej.nih.gov/ij/) [35]. For each analyzed protein, the quantified signal was normalized for the total nuclei number.

2.7. Statistical Analysis. All experiments were performed three times. The data are expressed as the means ± standard deviation, and statistical comparisons were performed with specific statistical packages (Prism v 7.00 GraphPad Software, San Diego, CA, USA). Differences were analyzed by one- or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Attenuation of ROS Production Induced by H_2O_2 in LC Pretreated H9c2 Cells. The diabetic heart usually has ROS levels that exceed normal quantities, and ROS overproduction likely contributes to cardiomyopathy [15, 36, 37]. After stimulus with 500 μM H_2O_2, 48 h pretreatment with 0.5 or 5 mM LC markedly reduced ROS levels with respect to the CONTR condition (Figures 2(b) and 2(c)). Furthermore, DAPI images showed that pretreated H9c2 cells with LC, in particular with 5 mM LC, showed a higher number of nuclei than the control condition (Figures 2(b) and 2(d)): These results suggested that LC pretreatment improved cellular survival after H_2O_2 injury.

3.2. LC Improves Antioxidant Response in H9c2 Cells under Hyperglycemic Condition. Signal transducer and activator of transcription 3 (STAT3) activation thought phosphorylation on Ser^{727} is an important protective mechanism to prevent ROS generation in the setting of oxidative stress [38–40]. Under normoxic conditions, the treatment with 0.5 or 5 mM LC for 48 and 72 h significantly enhanced myocardial STAT3 phosphorylation on Ser^{727} (Figure 3(a)).

Moreover, after 48 and 72 h of treatment, exposure of cells to 0.5 mM LC caused rapid increase of SOD2, a well-known antioxidant enzyme [41]. This effect was significantly augmented with the 5 mM LC dose (Figure 3(b)).

3.3. LC Downregulation of CaMKII Pathway. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a multifunctional serine/threonine protein kinase, is implicated in the pathogenesis of cardiac diseases [42, 43] promoting ROS overproduction [44, 45]. As shown by immunofluorescence analysis (Figure 4(a)), compared with untreated H9c2 cells, LC treatments decreased CaMKII and pCaMKII-positive cell numbers after 72 h of stimuli: immunofluorescence quantification shows a greater effect with the 5 mM LC dose. Furthermore, after 72 h, either 0.5 or 5 mM doses of LC significantly decreased the phosphorylation of CaMKII α isoform, as shown by Western blot analysis (Figure 4(b)).

3.4. LC Action on AMPK Protein Expression. 5′-AMP-activated kinase (AMPK) has become a strategic cellular target for the cure of cardiovascular disease correlated

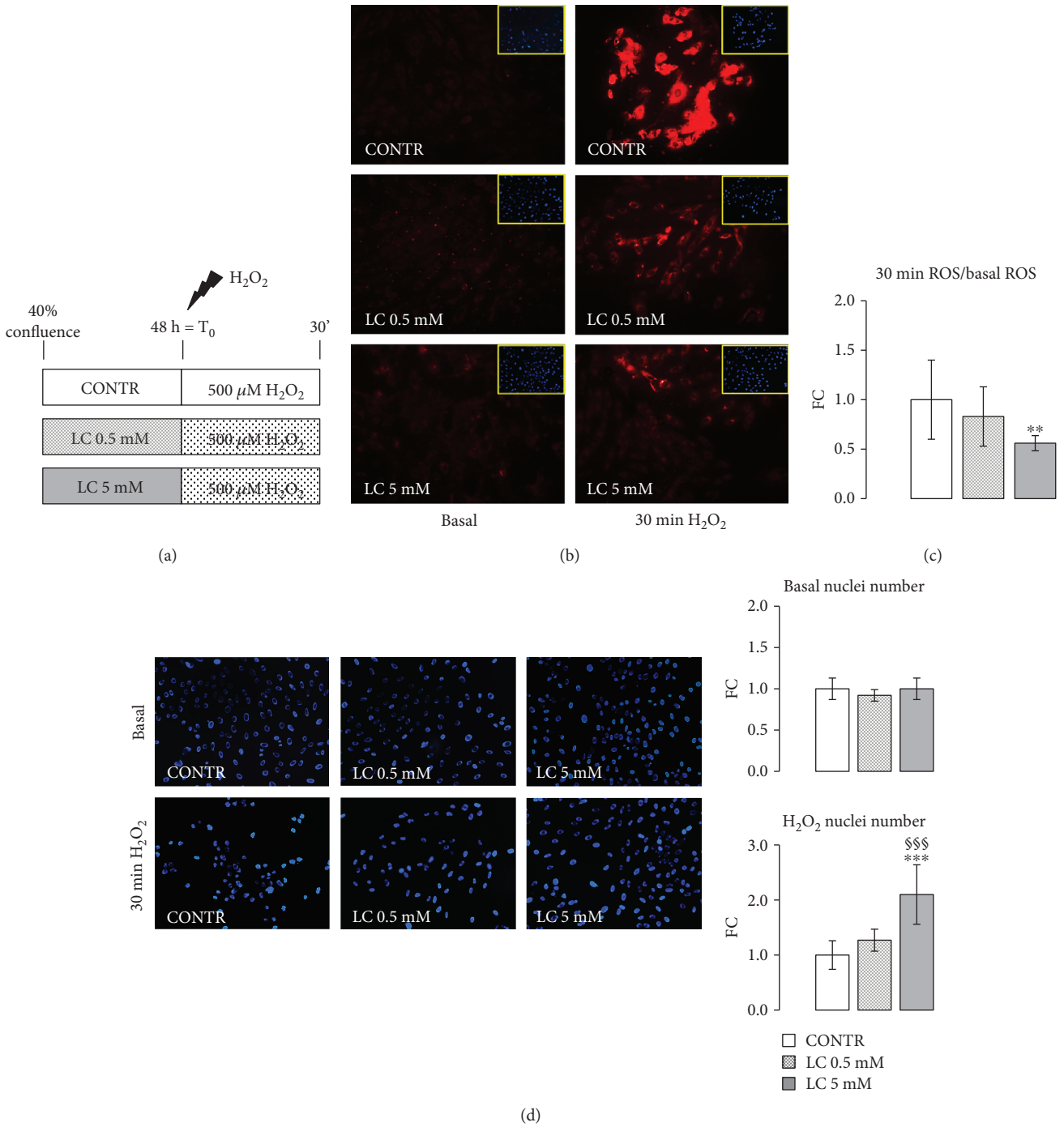

Figure 2: Attenuation of ROS production induced by H_2O_2 in LC-pretreated H9c2 cells. (a) Scheme of treatment: H9c2 cardiomyocytes were exposed to a stressful insult with 500 μM of H_2O_2 after pretreatment for 48 h with 0.5 or 5 mM LC. (b) Using the CellROX reagent assay, LC action was evaluated under oxidative stress conditions. The pretreatment with 0.5 or 5 mM LC causes a reduction in ROS production after stimulus with 500 μM of H_2O_2. (c) Quantification of ROS production. The quantified signal was normalized for the total nuclei number. From the DAPI images, it was possible to observe that the cells pretreated with both doses of LC show higher survival compared to the control cells. (d) Quantification of nuclei number pre- and after H_2O_2 injury in LC-pretreated H9c2 cells. Data are expressed as fold changes (FC) of mean ± SD. Significance: $^{**}p \leq 0.01$ vs. CONTR.

with DM [46–48]: it is likely that AMPK activity in the diabetic heart may ameliorate cardiac function. After LC stimuli, immunofluorescence for AMPK showed an increase in AMPK-positive cells in a dose- and time-dependent manner (Figure 5).

3.5. LC Action on Cardiomyocyte Viability. An uncontrolled consequence of sustained hyperglycemia is the induction of cardiomyocyte death that causes a loss of contractile units, which declines organ function and provokes hypertrophy of vital cardiomyocytes [49–51]. We

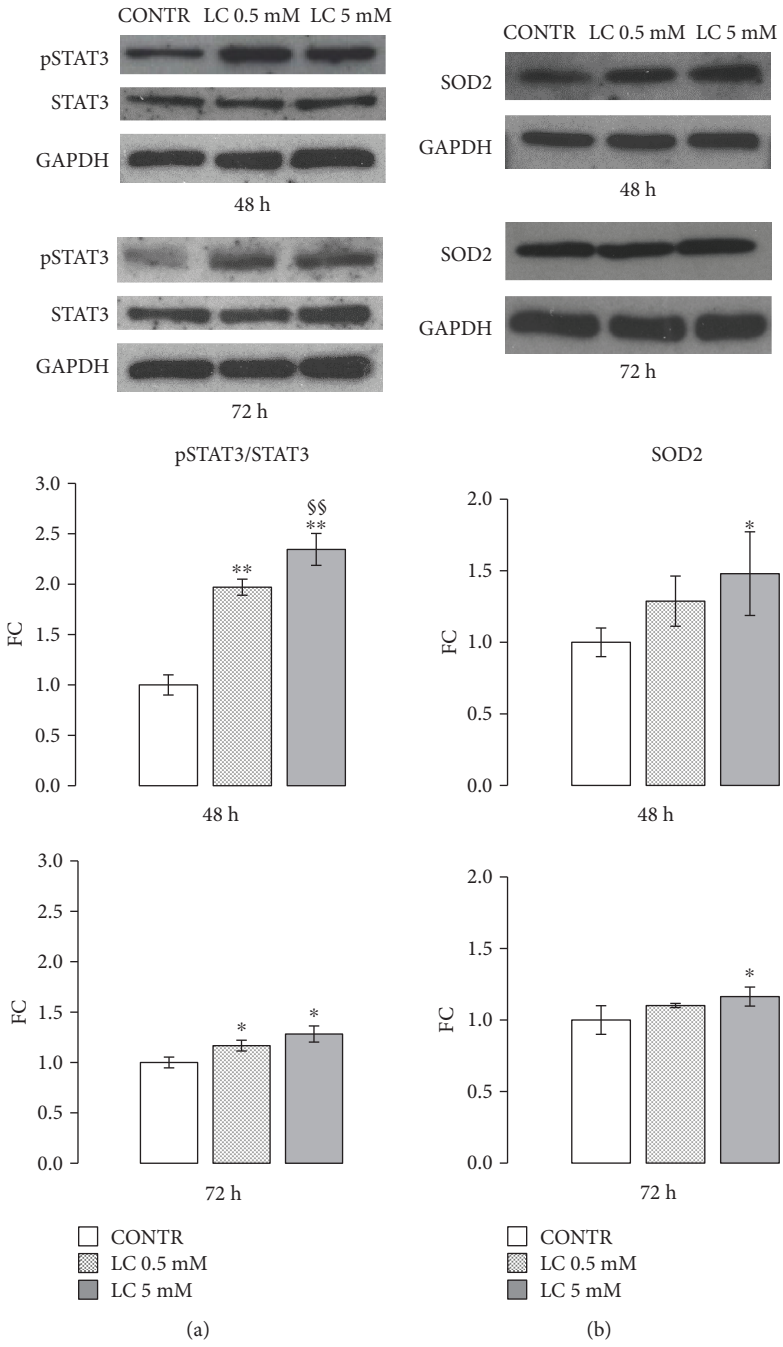

Figure 3: LC improves antioxidant response in H9c2 cells under hyperglycemic condition. (a) Representative Western blot and relevant quantification: LC stimuli for 48 and 72 h significantly enhanced STAT3 activation (ratio pSTAT3/STAT3). (b) Western blot analysis and relevant quantification: 5 mM LC improved SOD2 protein content in H9c2 cells after 48 and 72 h of treatment with respect to control cells. Data are expressed as fold changes (FC) of mean ± SD. Significance: $^{*}p \leq 0.05$ vs. CONTR; $^{**}p \leq 0.01$ vs. CONTR; $^{\S\S}p \leq 0.01$ vs. LC 0.5 mM.

investigated viability and morphologic features of H9c2 cells after exposure to 0.5 or 5 mM LC (Figure 6(a)). The growth curve showed that LC treatments did not induce a change of cellular proliferation with respect to untreated control cells (Figure 6(b)). Moreover, the viability graph showed the absence of cell mortality in all treatment conditions (Figure 6(b)). To support these data, phase contrast images, collected at day 3 of the growth curve, confirmed the absence of morphological changes in cells treated with 0.5 or 5 mM LC respect to control (Figure 6(c)).

In opposition to its antiproliferative functions, p21 can also play proproliferative and survival roles when it is localized in the cytosol [51]. Figure 6(c) shows that the protein content of p21 in cardiomyocytes treated with 0.5 or 5 mM LC was superimposable to control cells, and this result highlights that there is not p21 translocation from cytoplasm to nuclei.

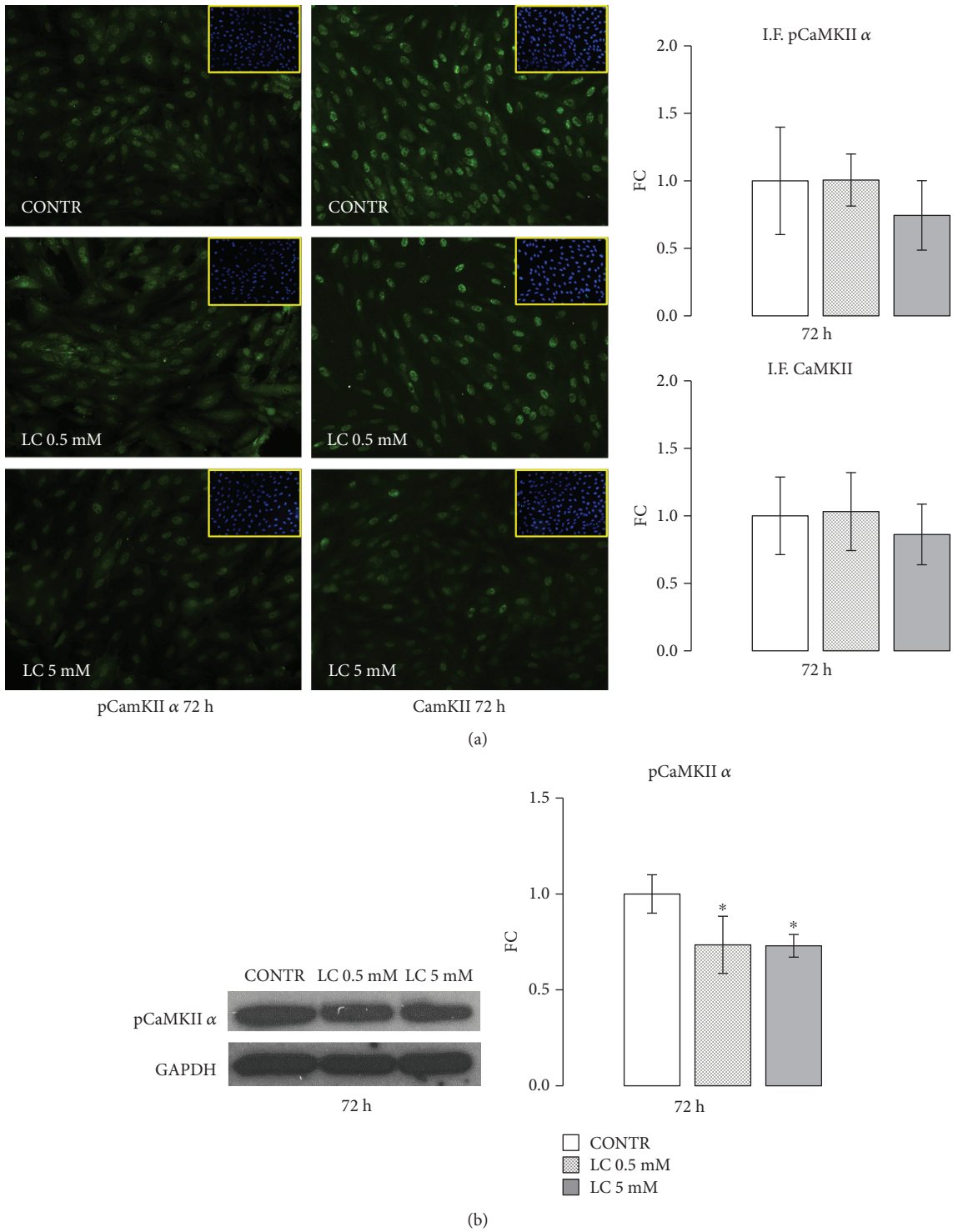

Figure 4: LC downregulation of CaMKII pathway. (a) LC treatments decreased CaMKII-positive cell number and its α phosphorylated form after 72 h of stimuli: this effect was more marked for the 5 mM LC dose. The quantified signal was normalized for the total nuclei number. (b) Representative Western blot and relevant quantification: LC stimuli for 72 h promote CaMKII α isoform activation. Data are expressed as fold changes (FC) of mean ± SD. Significance: $^{*}p \leq 0.05$ vs. CONTR.

4. Discussion

In the present study, we demonstrated that LC supplementation significantly decreases ROS production in cardiomyocytes during hyperglycemia (Figure 2). ROS generation induced by high glucose causes apoptosis of cardiac cells and important decrease in growth factor secretion [15, 16, 52–55]. In the heart microenvironment, the oxidative stress induced by hyperglycemia can lead to stem cell senescence which is characterized by the production and secretion of

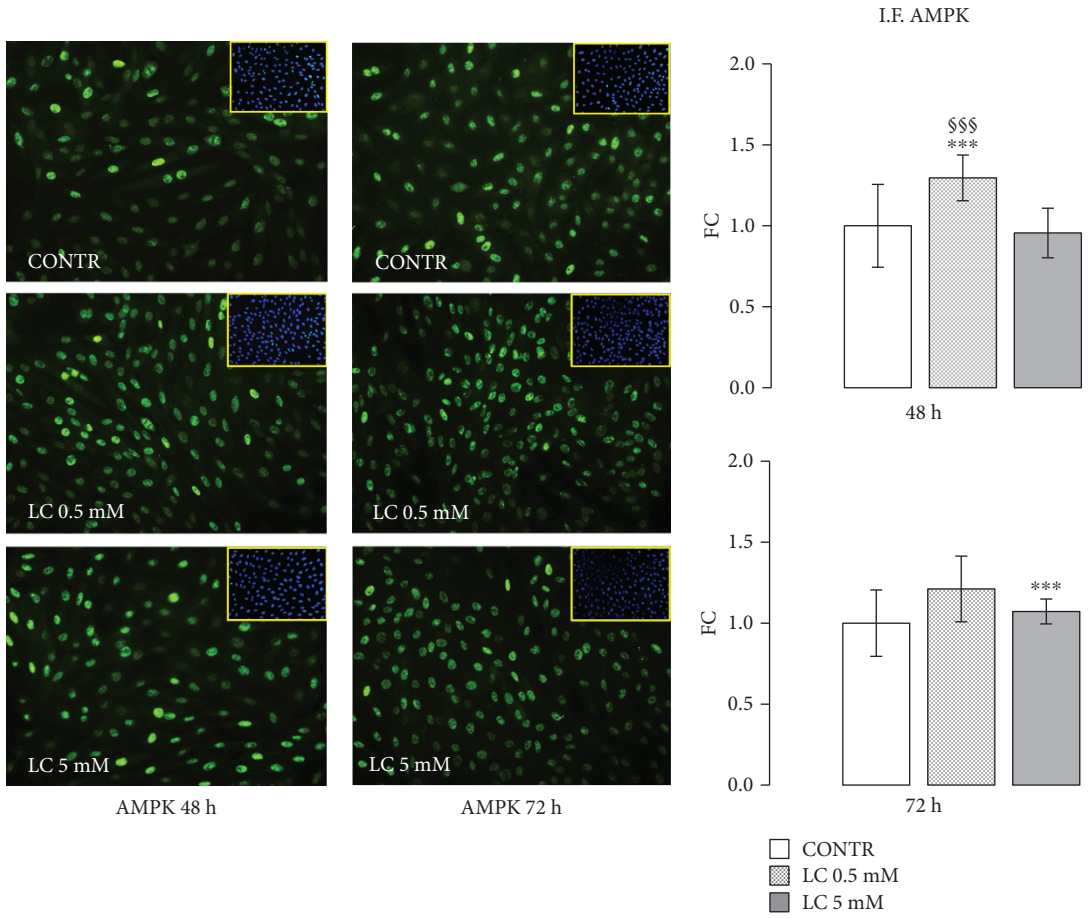

Figure 5: LC action on AMPK protein expression. Immunofluorescence assay and relevant quantification showed an increase in the number of AMPK-positive cells in LC condition at 48 and 72 h. The quantified signal was normalized for the total nuclei number. Data are expressed as fold changes (FC) of mean ± SD. Significance: $^{***}p \leq 0.001$ vs. CONTR; $^{§§§}p \leq 0.001$ vs. LC 5 mM.

soluble factor SASP (senescence-associated secretory phenotype). Those factors, responsible for the onset of chronic inflammation and oxidative stress, are considered the pathophysiological link between aging and diabetes in cardiovascular diseases [56].

The association hyperglycemia/ROS/cellular senescence represents a major cause of inefficiency of regenerative medicine [9, 10, 56, 57]. Further, clinical trials established that an altered glycemic control is equal to therapeutic failure [13, 14]. Our data point out that LC could counteract oxidative stress in the hyperglycemic condition. Those observations are consistent with several previous works that reported that LC stimuli decrease ROS generation in the skeletal muscle, bone and cardiac cells grown under normoglycemic condition [26, 27, 58, 59]. Furthermore, as shown in Figure 6, both doses of LC did not modify the H9c2 growth rate and did not induce cellular damages.

Studying the mechanism by which LC may modulate ROS formation, we observed an increase in the serine 727 (Ser^{727}) phosphorylation of STAT3, after 48 and 72 hours of treatment (Figure 3(a)). Recent evidence stresses the critical role of STAT3 in modulating mitochondrial respiratory chain function and ROS production [39, 40]. Serine 727 (Ser^{727}) phosphorylation has a primary part in STAT3 influence on mitochondria: its decrement is associated with the development of cardiac hypertrophy and dilated cardiomyopathy [60]. Moreover, several data showed that STAT3 conserves complex I activity in ischemic condition enhancing cell viability and plays an important role in heart protection from chronic stress induced by hyperglycemia [61]. STAT3 activation promotes the expression of SOD2, the principal enzyme effective in reducing mitochondrial oxidative species [41, 62]. LC treatment significantly increased SOD2 expression in *in vitro* cardiomyocytes, confirming LC capability to counteract oxidative stress in the hyperglycemic condition (Figure 3(b)).

Interestingly, a number of studies have indicated that CaMKII enhances mitochondrial dysfunction, ROS formation, and apoptosis [63], eventually causing cardiomyocyte death both following hyperglycemia [64, 65] and oxidative stress conditions [43, 45, 66].

Hyperglycemic cardiac cells are characterized by a vicious circle between ROS/mitochondrial dysfunction/CaMKII that triggers cellular damages and apoptosis phenomena [42–44]. Taking into consideration that literature evidence, we investigated LC action on CaMKII protein content. As reported in Figure 4, LC treatments reduce CaMKII activation. Remarkably, recent experiments hypothesized

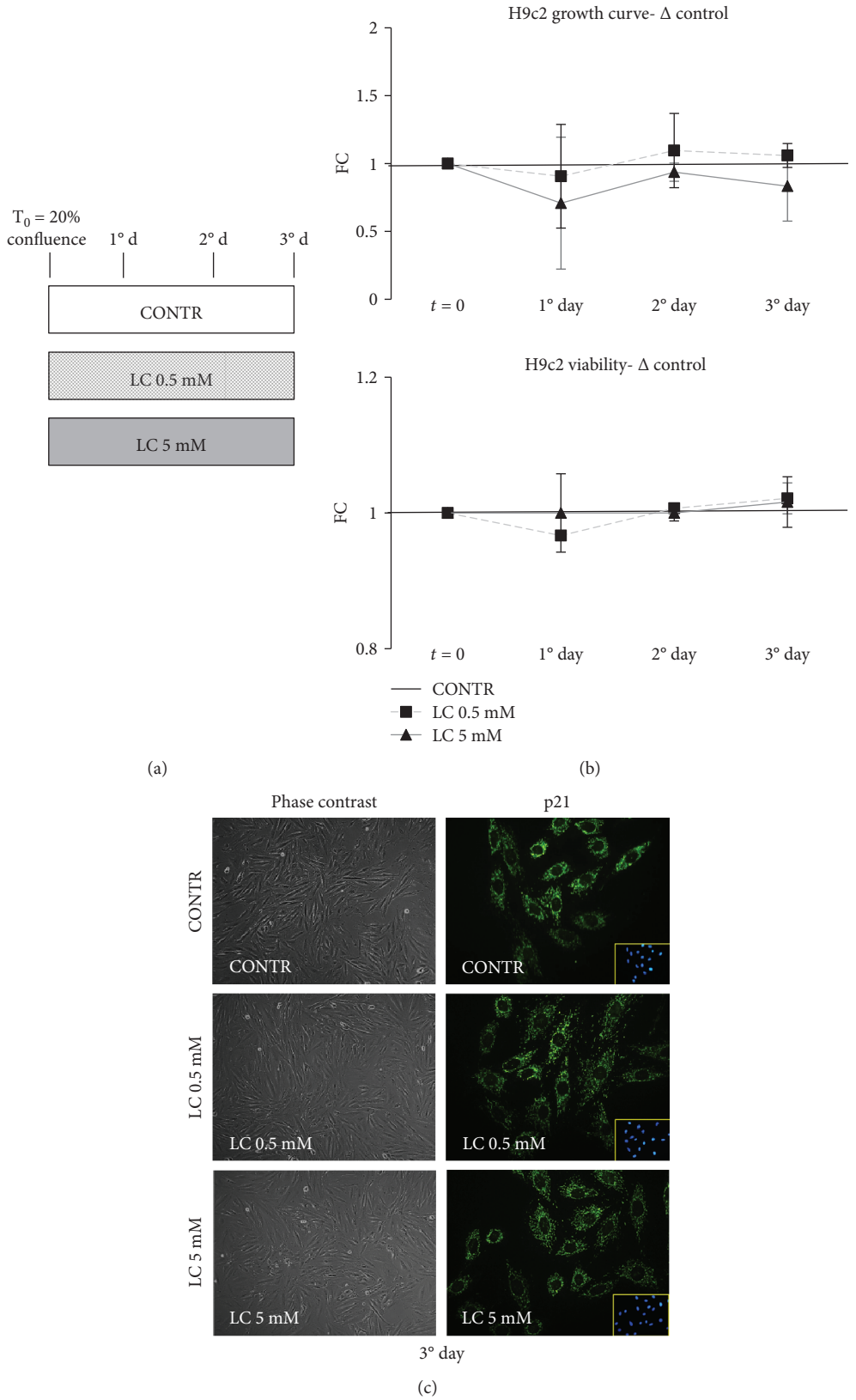

Figure 6: LC action on cardiomyocyte viability. (a) Experimental scheme for growth curve and viability determination. (b) 0.5 or 5 mM LC did not alter the H9c2 proliferative potential. Viability graph shows the absence of cell mortality in all treatment conditions. (c) Phase contrast images show how treatment with 0.5 or 5 mM LC do not modify the morphology of H9c2 cells. Furthermore, there is no p21 translocation from cytoplasm to nuclei following treatment with both doses of LC. Data are expressed as fold changes (FC) of mean ± SD.

Table 1: Summary of LC action on hyperglycemic cardiomyocytes (H9c2).

Hyperglycemic cardiomyocites (H9c2)					
	ROS	STAT3	CaMKII	AMPK	Cellular growth and vitality
L-Carnitine treatment	⇩	⇧	⇩	⇧	=

that CaMKII activation is related to Ca^{2+} movement from the endoplasmic reticulum to mitochondria.

Recently, LC was shown to promote Ca^{2+} availability which is needed for proliferation and differentiation of human osteoblast-like cells, via a depolarization of L-type calcium channels [59]. Despite the Ca^{2+} supply via the L-type channels being essential to ensure appropriate cardiac cell contraction, an excessive Ca^{2+} influx increases mitochondrial ROS production in cardiomyocytes under oxidative stress. Various authors suggested that cardiac damage, induced by CaMKII activation, is caused by an increase in L-type Ca^{2+} current [67].

LC capability of modulating CaMKII in cardiac cells could make LC a molecule appropriate for impeding the cross-talk between L-type Ca^{2+} channels and ROS production and restoring normal mitochondrial function in cardiac cells. Further investigations are necessary to unravel LC action on cardiac calcium channels.

AMPK represents a strategic target in cardiovascular alterations associated with DM. It is well known that impairment of AMPK activation characterizes hyperglycemic cardiomyocytes. In particular, during hypoxia/reoxygenation (H/R) injury in diabetic patients, AMPK deficiency is associated with an increase in ROS production and apoptosis [46, 47]. As shown in Figure 5, LC stimuli enhanced AMPK protein synthesis, suggesting that LC not only could ameliorate the oxidative microenvironment but also could improve metabolic functions of cardiac cells. With this in mind, future studies could investigate LC's possible action on the protein kinase C (PKC) signaling pathway, implicated in diabetic damage. As known, in hyperglycemic condition, the decrease in AMPK activity is associated with an increase of diacylglycerol (DAG) production that activates PKC and NADPH-oxidase causing an abnormal production of ROS [68, 69]. Then, LC ability to stimulate AMPK activation and to regulate the production of acyl-CoA could counteract hyperinsulinemia-correlated oxidative stress.

Whereas the higher risk of developing atrial fibrillation (AF) in diabetic subjects compared to healthy ones is associated with mitochondrial ROS overproduction, ATP depletion, and abnormal calcium homeostasis [70, 71], the key modulator role shown by LC suggests the potential use of this micronutrient as adjuvant therapy in different cardiac pathologies associated with DM.

In conclusion, LC described effects could be useful in association with other cardioactive drugs. Ranolazine is an antianginal drug with hypoglycemic action used in AF treatment, which was shown to enhance skeletal muscle differentiation [35]. In cardiac regenerative medicine, antioxidative LC action, in association with ranolazine, might lead to the improvement of the hyperglycemic-oxidative microenvironment, prevention of apoptosis, and preservation of cell viability. Equally interesting could be the synergistic effect of LC with allopurinol [72] or the α-lipoic acid antioxidant [73] in the prevention and treatment of AF. Remarkable therapeutic perspectives could arise from the association of LC with new hypoglycemic drugs, such as incretins, whose effects on the hyperglycemia control, inflammation, and atherosclerotic plaque progression have recently been demonstrated [74, 75].

Taken together, our results argue that the use of LC as a coadjuvant in therapeutic treatments is very promising, although extensive studies focused on the determination of the effective LC dose and route of administration in humans should be undertaken. Anyway, LC doses used in our study (0.5–5 mM) are compatible with ranges used in humans receiving LC therapy [76, 77].

5. Conclusion

The results of this work (Table 1) provide, for the first time, fundamental *in vitro* cellular evidence that treatment with LC could be a potential strategy to improve the cardiac oxidative microenvironment caused by hyperglycemia.

According to the findings of the present study, demonstrating the LC ability to decrease production of ROS, activate STAT3 and AMPK, and downregulate CaMKII, LC could represent a potential adjuvant therapeutic strategy in diabetic cardiac regenerative medicine.

This adjuvant therapy might be recommended for diabetic patients with high risk of cardiac injury as it exerts a cardioprotective effect by restoring the microenvironmental equilibrium by strengthening cardiomyocytes' defense mechanisms through the stimulation of endogenous antioxidants (SOD2).

Further, *in vitro* and *in vivo* studies on this topic are essential to improve the knowledge on the effects of LC in cardiac diseases associated with diabetes and on its potential synergic action with other antioxidative and hypoglycemic agents.

Data Availability

The authors confirm that the data supporting the findings of this study are openly available within the article.

Conflicts of Interest

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

Authors' Contributions

FV, PS, and AM are coauthors and contributed equally to this work.

Acknowledgments

This study was partially supported by the Ricerca Corrente funding from the Italian Ministry of Health to IRCCS Policlinico San Donato.

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Hindawi
Journal of Diabetes Research
Volume 2018, Article ID 5205126, 9 pages
https://doi.org/10.1155/2018/5205126

Review Article

Implications of the Intestinal Microbiota in Diagnosing the Progression of Diabetes and the Presence of Cardiovascular Complications

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Received 10 August 2018; Revised 6 October 2018; Accepted 21 October 2018; Published 12 November 2018

Guest Editor: Gaetano Santulli

The prevalence of diabetes is steadily rising, and once it occurs, it can cause multiple complications with a negative impact on the whole organism. Complications of diabetes may be macrovascular: such as stroke and ischemic heart disease as well as peripheral vascular and microvascular diseases—retinopathy, nephropathy, and neuropathy. Key factors that cause cardiovascular disease in people with diabetes include hyperglycemia, dyslipidemia, obesity, insulin resistance, inflammation, hypertension, autonomic dysfunction, and decreased vascular response capacity. Microbes can be considered a complex endocrine system capable of ensuring the proper functioning of the body but are also responsible for the development of numerous pathologies (diabetes, coronary syndromes, peripheral arterial disease, neoplasia, Alzheimer's disease, and hepatic steatosis). Changes in the intestinal microbiota may influence the host's sensitivity to insulin, body weight, and lipid and carbohydrate metabolism. Dysbiosis causes activation of proinflammatory mechanisms, metabolic toxicity, and insulin resistance. Trimethylamine N-oxide (TMAO) is a microbial organic compound generated by the large intestine, and its concentration increases in the blood after ingestion of foods rich in L-carnitine and choline, such as red meat, eggs, and fish. The interest for TMAO in cardiometabolic research has recently emerged, given the preclinical evidence that reveals a link between TMAO, diabetes, and cardiovascular complications. Intestinal microbiota can be modulated by changing one's lifestyle but also by antibiotic, probiotic, prebiotic, and fecal transplantation. The purpose of this article is to highlight issues related to the involvement of microbiota and trimethylamine N-oxide in the pathogenesis of diabetes mellitus and cardiovascular disease. Better appreciation of the interactions between food intake and intestinal floral-mediated metabolism can provide clinical insights into the definition of individuals with diabetic risk and cardiometabolic disease as well as potential therapeutic targets for reducing the risk of progression of the disease.

1. Definition

Microbiota is part of a complex system that includes all microorganisms, cells, antimicrobial peptides, luminous compounds, and all interactions between them [1, 2].

The microbiota is involved in numerous activities such as vitamin production, regulation of gene expression, fight against pathogenic bacteria, absorption of nutrients, and regulation of metabolic disorders [3].

Intestinal microbiota is influenced by factors such as genetics, lifestyle, diet, and antibiotherapy [2, 4, 5].

Microorganisms such as bacteria, viruses, and fungi survive in the gastrointestinal tract. The intestinal microbiota is the result of the complex interaction between the environment and host genetics; diet is the component that modulates the intestinal bacterial activity. An imbalance of intestinal homeostasis causes the internal dispersion of bacterial fragments and promotes intestinal permeability and bacterial and circulating endotoxin translocation, which initiates inflammation in tissues responsible for insulin metabolism thus causing insulin resistance [1, 6]. Dysbiosis also plays an important role in the pathogenesis of cardiovascular and metabolic diseases [7, 8].

The intestinal microbiota can be considered a gate that modulates the transition into cardiometabolic diseases that involve the hepatobiliary tract [4, 7].

2. Microbiome Composition

Our body is colonized by a series of microbiota, primarily bacteria, which exist in a symbiotic relationship with the host and play an important role in maintaining the homeostasis of the host [8]. The intestinal microbe is comprised of billions of cells, of which the most important are Gram-positive bacteria belonging to the phyla Firmicutes and Actinobacteria and also to the genera Clostridium, Bifidobacterium, Lactobacillus, Ruminococcus, and Streptococcus and Gram-negative bacteria belonging to the genera Bacteroides, Prevotella, and Akkermansia [2].

The link between microbiota compounds and the host's immune system is supported by a series of molecules and signaling processes that can affect the intestine, liver, brain, and other organs [9]. On the other hand, the intestinal immune system plays a significant role in the exposure of bacteria to host tissues, causing stratification of intestinal bacteria on the lumbar side of the epithelial barrier, and controls the composition of the intestinal microbiota [4]. Residual bacteria provide signals that favor the development of a normal immune system and regulate the resulting immune responses. Changing these can cause significant repercussions on the host's health [9]. The disruption of the intestinal microbe may also be associated with numerous pathologies: metabolic syndrome, obesity, diabetes, renal disease, cardiovascular disease, neoplasia, and Alzheimer's disease [2]. Clostridium species correlate negatively with glucose, HbA1c, and insulin levels, whereas Lactobacillus species correlate positively with glucose and HbA1c levels [10]. It has been demonstrated that a higher blood glucose concentration can be predicted by reducing the proportion of anaerobes, especially Bacteroides [11]. Markers of glucose metabolism disorders (e.g., insulin and insulin resistance-HOMA-IR) are commonly associated with the microbial genotype, suggesting that people with fewer genotypes are predisposed to metabolic disorders and secondarily to diabetes [11].

The intestinal microbiota influences the host's health by the intestinal immune response. L-Tryptophan plays a significant role in maintaining the balance between intestinal microfibres and immune tolerance. Modification of the tryptophan metabolism influences the intestinal microbiota. Bacterial metabolites (indole, indolic acid, and tryptamine) and endogenes (serotonin, melatonin, and kynurine) influence the microbial metabolism, microbiota composition, and host immune system [9] (Figure 1).

Indoxyl sulfate is a metabolite of tryptophan-derived intestinal bacteria. Several studies show that indoxyl can affect the functions of the circulatory system by lowering NO (nitric oxide) production, increasing the production of reactive oxygen species, and promoting cardiac interstitial fibrosis [12].

Indole is a tryptophan metabolite that can regulate bacterial motility, antibiotic resistance, virulence, and intestinal biofilm formation. Indole catabolism is supported by tryptophanase, which can be induced by tryptophan or suppressed by glucose. Bacterial species, including *E. coli*, Proteus vulgaris, Paracolobactrum coliforme, Achromobacter liquefaciens, and Bacteroides spp., are capable of producing indole [9].

On the other hand, intestinal microbiota can use nutrients such as tryptophan, an essential amino acid, thereby reducing the supply of substrates for the endogenous synthesis of vital host compounds [13].

Indole administration can alleviate gastrointestinal tract damage induced by nonsteroidal anti-inflammatory drugs (NSAIDs), modulating inflammation mediated by innate immune responses and changes in intestinal microbial composition [9]. It has been shown that indole promotes the functions of the intestinal cell epithelial barrier by fortifying tight epithelial junctions between cells via receptor X (PXR), which may contribute to inflammation resistance. Indole can also enhance glucagon-like peptide-1 (GLP-1) secretion, an incretin with profound influences on host metabolism [4, 9].

For normal cells, exposure to indole can strengthen the mucosal barrier and mucin production by inducing expression of associated genes, thus increasing resistance to pathogenic invasion. For inflammatory cells, indole exposure can suppress the activation of NF-κB chemokine production and, at the same time, increase the production of anti-inflammatory cytokines, thus improving inflammation and damage [9].

Most of the compounds derived from the intestine first enter the liver; systemic effects can also be exerted by hepatic metabolites of compounds derived from intestinal bacteria or changes in liver metabolism. To access the circulation, molecules derived from intestinal bacteria have to cross the intestinal barrier (GBB) [10, 13]. Some clinical and experimental studies show that cardiovascular disease may affect GBB function and that GBB permeability may be a new marker

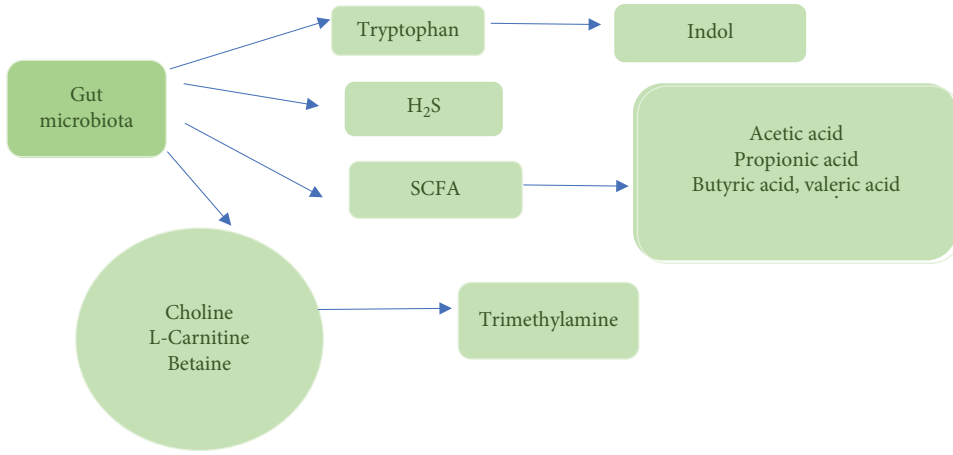

Figure 1: Gut microbiota metabolites.

in CVD. In this case, a key factor for the proper functioning of GBB is proper blood perfusion through the intestines [13].

Multiple studies suggest that intestinal microbiota could produce biologically active compounds that enter the circulation and affect circulatory system homeostasis. To enter the circulation, intestinal bacterial metabolites have to pass through the intestinal barrier (GBB). The integrity and permeability of the intestinal barrier depend on numerous factors, including intestinal blood flow [14].

The intestinal blood barrier is made up of several layers. The internal or mucosal layer prevents pathogens from adhering to epithelial cells. The physical barrier consists of a single layer of enterocytes connected by tight junctions, which play a crucial role in the selectivity of the intestinal barrier [15].

Intestinal bacteria produce many vital nutrients for human homeostasis, such as vitamins K and B and SCFA which contribute to the transformation and degradation of biliary acids and steroids [13].

Intestinal metabolites such as hydrogen sulfide (H2S), SCFA, indole, or trimethylamine may exert effects on circulatory system homeostasis and on nerve and humoral control. Short-chain fatty acids, including acetic, propionic, butyric, and valeric acids, are formed from carbohydrates by bacterial fermentation [14] (Figure 1). The amount of SCFA may have vasorelaxant effects on the arterial resistance in the colon, improving microcirculation. SCFA depends on the composition of intestinal bacteria, diet, and intestinal transit time and plays a local role as an energy source for intestinal cells suppressing the growth of pathogens by reducing the pH in the intestines. There is also some evidence that SCFA derived from intestinal bacteria can affect blood pressure [13].

Short-chain fatty acids (SCFA), produced by bacterial fermentation in the colon, contribute to a significant proportion of the daily energy requirement. SCFA, especially butyrate and propionate, play an important role in differentiating regulatory T cells and regulating immunity in the intestinal tract. Increased production of acetate by intestinal microbiota could lead to the activation of the parasympathetic nervous system, which promotes increased secretion of insulin stimulated by glucose, hyperphagia, and obesity [16].

Intestinal bacteria produce many biologically active molecules, some of which play a role in regulating the circulatory system and energy balance. Besides numerous metabolites, intestinal flora produces methylamine, including TMA, dimethylamine, and monomethylamine. The intestinal barrier is considered a functional, immunological, and anatomical unit, separating the intestinal lumen from circulating blood, preventing bacterial adherence and transport regulation. Methanolamine and other intestinal metabolites reach almost all tissues as small molecules thus affecting both neurohormonal and peripheral regulatory mechanisms [17].

A fat-rich diet causes bowel dysbiosis and reduces intestinal integrity. Recent studies have shown that dysbiosis may contribute to the development of inflammation and subsequently the progression of cardiovascular disease (CVD) by promoting two major risk factors—arterial hypertension and atherosclerosis [7, 10].

TMAO precursors such as choline, L-carnitine, γ-butyrobetaine, phosphatidylcholine, betaine, glycerophosphine, and crotonobetaine are metabolized by the intestinal microbiota to produce trimethylamine which is then further metabolized to TMAO by the monooxygenase 3 enzyme (FMO3) [18]. Metabolizing TMAO by FMO3 was linked to insulin sensitivity and glucose metabolism [19]. Several studies have also suggested a significant role of choline in regulating insulin resistance and glucose metabolism. Diet significantly affects the intestinal microbiota and the production of TMAO [20].

FMO3 (flavin monooxygenase 3) is the preponderant enzyme in the liver, and flavin monooxygenase 1 and flavin monooxygenase 2 (FMO1 and FMO2, respectively) can also cause TMAO oxidation. In some patients with FMO3 gene mutation, accumulation of trimethylamine (TMA) spreads in the body and is released through sweating and breathing, resulting in fish smell syndrome, a genetic disease [20].

The plasma level of TMAO in the human organism is in the range of 0.5-10 μmol/L. Recently, a number of clinical trials have indicated a possible positive correlation between increased plasma TMAO and an increased risk of cardiovascular disease [13].

Choline is an essential nutrient which is both synthesized endogenously and obtained from various animal and plant

products [21, 22]. Food-derived choline is generally metabolized in the liver and is involved in various biological processes, synthesis of acetylcholine neurotransmitter, lipoprotein, and membrane phospholipids [23]. Betaine is the direct oxidation product of choline, which is a metal donor in homocysteine remethylation, and plays an important role in maintaining stability and cellular volume [22]. Together, choline and betaine have been recognized as achieving hepatoprotection and improving insulin resistance [23].

The intestinal microbiota through enzyme activity can turn choline into trimethylamine (TMA), a harmful metabolite known for its strong ammonia smell. TMA is absorbed and transmitted to the liver, where it can be rapidly detoxified by monooxygenase 3 (FMO3) to be transformed into trimethylamine N-oxide (TMAO) [23].

Significant conversion of choline into TMA by the intestinal microbiota may, however, reduce the bioavailability of choline, which could affect the secretion of very-low-density lipoproteins, with increased accumulation of triglycerides in the liver and could stimulate hepatic steatosis [22–24].

Blood TMAO levels depend on many factors, including diet, intestinal barrier permeability, liver enzyme activity and methylamine excretion rate, composition, and activity of the intestinal microfibres [25]. Changes in intestinal microbiota may influence the host's sensitivity to insulin with the onset of diabetes. Several studies have shown that the level of TMAO is significantly associated with the risk of type 2 diabetes [26, 27].

Thus, FMO3 is suppressed by insulin, and also FMO3 levels are elevated by glucagon secretion from pancreatic α cells to stimulate an increase in blood sugar [28]. Both glucagon suppression and insulin resistance are correlated with weight loss and act together to improve glucose homeostasis following a dietary restriction with body mass loss [26, 29]. A high-fat diet has led to changes in the intestinal microbial composition causing insulin resistance [30].

TMAO may cause inflammation of the adipose tissue with disruption to the insulin signaling pathway. This mechanism plays an important role in the emergence of insulin resistance and subsequently in the evolution towards diabetes [31–33]. The increase in TMAO levels may result from dietary differences; intestinal microbiota plays an important role in varying TMAO levels. L-Carnitine is essential for the mitochondrial metabolism of long-chain fatty acids, and some studies have provided evidence of glycemic control and plasma lipid control following L-carnitine administration in type 2 diabetes [29]. However, other studies demonstrate the increased risk of diabetic complications in patients with higher circulating L-carnitine concentrations. The role of L-carnitine in cardiovascular health was recognized after discovering the proatherogenic nature of TMAO and its relationship to L-carnitine metabolism [26].

TMAO produces a proatherogenic macrophage phenotype that affects the metabolism of cholesterol and sterol in macrophages, intestines, and liver. Studies have shown that diabetes and body mass index (BMI) are associated with higher levels of TMAO [27, 28]. After ingestion of phosphatidylcholine or L-carnitine, circulating TMAO levels increase in 4 to 8 hours and normalize after 24 hours depending on renal clearance [30]. Trimethylamine N-oxide (TMAO), which is derived from intestinal metabolite-derived metabolites, is possibly linked to diabetic, atherosclerotic, and cardiovascular risk [25]. The circulating TMAO levels are elevated and associated with the severity of the disease and with patients with atherosclerosis, chronic kidney disease, and peripheral arterial disease [7, 8]. Previous studies have shown that the bacterial species belonging to the families Clostridiaceae and Peptostreptococcaceae have been associated with increased blood levels of TMAO in humans [8].

TMAO generates atherosclerosis, perhaps by forming foam cells in the arterial wall. High levels of TMAO affect lipid metabolism, and inflammatory response promotes endothelial dysfunction and exacerbation of platelet reactivation and stimulates thrombosis. This highlights the importance of this molecule for cardiovascular complications [28].

TMAO activates in vascular smooth muscle cells and endothelial cells MKKK (mitogen-activated protein kinases), and the isolation of nuclear factor-κB (NF-κB) leads to increased expression of inflammatory genes and adhesion of endothelial cells to leukocytes. TMAO in vivo can increase the receptor expressed on CD36 and SR-A1, leading to the formation of foam cells, by a greater absorption of modified macrophage LDL. Furthermore, TMAO increases calcium concentration in the endoplasmic reticulum in the platelets, which consequently leads to platelet aggregation and thrombosis with increased risk of acute coronary syndromes [20] (Figure 2).

TMAO activates prothrombotic pathways by rapidly increasing the release of calcium ions (Ca^{2+}) resulting in the activation of certain platelet stimuli. In endothelial cells and smooth muscle cells, TMAO rapidly activates the mitogen-activated protein kinase and the activated B cell activated kappa amplifier factor, which in turn favors the expression of adhesion molecules such as E-selectin [20]. TMAO can regulate and differentiate monocytes into foam cells and macrophages. TMAO can initiate profibrotic processes in the heart and kidneys by transforming the phospho-SMAD3 growth factor-β signaling axis [20, 34]. The association of all these complex cellular mechanisms accelerates atherosclerosis and thrombotic vascular disease and leads to secondary renal insufficiency [35]. Therefore, understanding the molecular mechanism of action of TMAO and discovering new mechanisms and receptors by which TMAO may lead to its adverse effects will have a much wider implication in clarifying its role in pathogenesis in humans [20, 35].

Increased levels of TMAO and choline are associated with low levels of HDL-cholesterol and plasma phospholipids [29]. Increased concentrations of TMAO in the blood influence the activity of intestinal microbiota and the permeability of the intestinal barrier and determine the activity of liver enzymes. There was a direct relationship between plasma TMAO concentrations, diabetes mellitus, acute coronary syndromes, and peripheral vascular disease [25, 36].

Furthermore, some experimental studies show that TMAO should affect lipid and hormone homeostasis, providing indirect evidence for the possible contribution of TMAO to the development of CVD. It has been found that TMAO could lead to decreased beta-oxidation of fatty acids

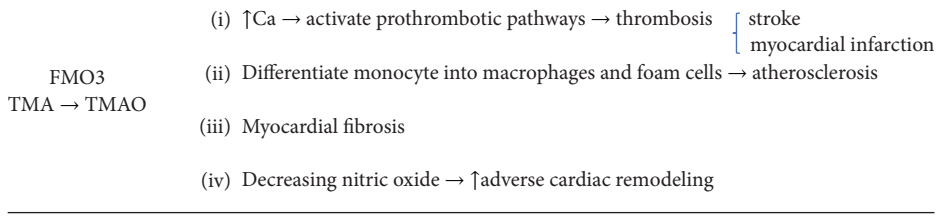

Figure 2: Effect of TMAO on cardiovascular disease.

through cardiac muscle cells. In addition to TMA, other metabolites have also been reported to play a role in the pathology of many diseases [16].

Indoxyl sulfate is produced by intestinal microbial tryptophanases that convert food tryptophan to indole, which is then transformed into indoxyl and indoxyl sulfate in the liver. It has been shown that indoxyl sulfate may have proinflammatory and prooxidant effects on cardiomyocytes and cardiac fibroblasts [16].

It is believed that complex molecules mediate intracellular and extracellular signaling, but it also appears that gaseous molecules, later referred to as "gas transmitters," play an essential role in maintaining the body's homeostasis. The gas transmitters include carbon monoxide (CO), hydrogen sulfide (HS), and nitric oxide (NO), the cytotoxic molecule produced by phagocytic leukocytes [37].

The intestinal microbe uses sulfur-containing compounds to produce hydrogen sulfide. Hydrogen sulfide is an important biological mediator that is involved in various physiological processes, including blood pressure regulation [13, 16]. Moreover, phenylacetylglutamine is a product that is formed by the conjugation of phenylacetate and glutamine. Increased serum concentrations of phenylacetylglutamine should be a strong and independent risk factor for overall mortality and cardiovascular disease. Further studies are needed to elucidate the causal relationship between these metabolites and CVD [16].

3. Diabetes, Cardiovascular Diseases, and Microbiota

When the pancreas does not produce enough insulin or when the body cannot use insulin produced by the pancreas, diabetes can occur [38].

Diabetes mellitus is associated with an alteration of interdependent metabolic pathways (phospholipids, lipids, and methylation) and also with diseases such as retinopathy, nephropathy, neuropathy, and heart failure [36, 39].

Diabetes mellitus is a major risk factor for cardiovascular disease (CVD), which is the most common cause of death among adults with diabetes. The link between hyperglycemic status and microvascular disease is much more common than the link between hyperglycemic status and macrovascular disease, with a 37% increase in risk of renal failure or retinopathy [40].

Prevalence of diabetes increases with age. Diabetes patients are at a higher risk of developing cardiovascular disease. Inflammation and oxidative stress have a role in the mechanisms underlying cardiovascular disease and other complications in the development of diabetes [41]. Diabetics have a two to four times higher risk of developing cardiovascular complications and premature death. Myocardial ischemia is frequently asymptomatic in patients with diabetes and is associated with an unfavorable prognosis [42].

Diabetes causes various microvascular complications, such as autonomic and peripheral neuropathy, nephropathy, and retinopathy, and these complications are correlated with adverse cardiovascular effects [43].

Atherosclerosis of the large arteries and coronary arteries leads to macrovascular complications such as stroke, ischemic heart disease, and peripheral vascular disease. Atherosclerosis of small arteries causes diabetic nephropathy and is related to cardiovascular morbidity. Diabetes, regardless of its effect on atherosclerosis, is associated with changes in cardiac structure and function leading to myocardial dysfunction, called "metabolic cardiomyopathy" [42].

The presence of a metabolic syndrome exposes patients to an increased risk of cardiovascular complications. Moreover, in addition to classical risk factors, there are other unconventional factors that cause vasoconstriction and thrombosis, endothelial dysfunction, inflammation, oxidative stress, and vascular wall abnormalities that also contribute to an increased cardiovascular risk [40]. When the glycemic level falls below 70 mg/dL, autonomic nerve activation occurs. This may produce symptoms such as tremor, tachycardia, diaphoresis, anxiety, hunger, and headache [39, 40]. There are several mechanisms through which hypoglycemia could promote adverse cardiovascular effects in high-risk individuals. Hemodynamic changes following autonomous self-induced hypoglycemia include increased systolic blood pressure, heart rate, myocardial contractility, and cardiac output [36].

These effects can exacerbate ischemia. Hypoglycemia, as a complication of long-term diabetes, has also been associated with a prolonged QT interval. The relationship between hypoglycemia, autonomic neuropathy, and cardiac repolarization may contribute to arrhythmias and the risk of sudden death in people with diabetes [38]. Finally, hypoglycemia may have adverse effects on endothelial function, platelet reactivation, and coagulation cascade, and this increases blood viscosity and decreases serum potassium levels [40].

Concerns about cardiovascular complications associated with type 2 diabetes have traditionally focused on atherosclerotic vasculo-occlusive events such as myocardial infarction, stroke, and limb ischemia [41]. However, one of the most common and most serious cardiovascular

disorders in patients with diabetes is cardiac insufficiency. Cardiac insufficiency and diabetes are physiologically related [42, 43]. Type 2 diabetes and heart failure have a common insulin resistance characteristic and are accompanied by the activation of the cascade of neurohormonal systems: norepinephrine, angiotensin II, aldosterone, and neprilysin. The two diseases overlap; diabetes is present in a proportion of 35-45% of patients with chronic heart failure, regardless of whether they have a reduced or conserved ejection fraction [44].

Reduced blood flow from the intestinal endothelium in patients with heart failure is due to a decreased cardiac output which causes intestinal wall ischemia, leading to disruption of the intestinal barrier function, increasing permeability [40, 43].

Systemic congestion in patients with heart failure may also cause edema of the intestinal wall, resulting in increased intestinal permeability. Thus, a translocation of endotoxins, microbial metabolites, and microbial components produced by Gram-negative bacteria entering the systemic circulation is determined at the intestinal level. These processes may further activate cytokines and may generate systemic inflammation that contributes to the progression of heart failure [7]. There is evidence of chronic heart failure (CHF) and gastrointestinal (GI) involvement in this syndrome. It is known that cytokine activation occurs in patients with chronic NYHA class III-IV heart failure, with both clinical severity and prognosis. It has been suggested that endotoxin may be an important stimulant for the production of cytokines in patients with chronic heart failure by its action on mononuclear cells [43–45]. According to this hypothesis, endotoxin enters the circulation through bacterial translocation in the intestine. The two main factors are intestinal edema and hypoperfusion. The finding that patients with edematous heart failure decompensation have elevated levels of endotoxin normalizing after diuretic treatment tends to suggest that edema of the intestinal wall may contribute to endotoxemia. In addition, there is evidence that intestinal hypoperfusion may result in mucosal ischemia, which may lead to increased intestinal wall permeability. Furthermore, the intestine may play an important role in the development of cardiac cachexia [45].

Bowel dysfunction and dysbiosis may contribute to metabolic disease. Diabetes mellitus and cardiovascular disease can compromise intestinal function by producing macro- and microangiopathy. In cardiac failure, there is centralization of circulation that further reduces intestinal perfusion. Intestinal ischemia leads to the progressive deterioration of the connections between enterocytes and an accelerated passage through the blood barrier [15].

Excessive intake of salt is considered a cardiovascular risk factor. Studies suggest that metabolites from intestinal bacteria, such as trimethylamine N-oxide (TMAO), are considered to be a potential marker of cardiovascular diseases and may affect homeostasis [12, 16]. Increased evidence suggests that homeostasis may very much depend on a mutualist relationship with intestinal bacteria and that CVD is associated with intestinal microbial dysbiosis. Research has shown that high salt intake is associated with increased TMAO in plasma and reduced urinary excretion of TMAO. Furthermore, it has been found that increased salt intake affects the intestinal microbial composition [12].

High blood pressure is a major risk factor for heart failure, coronary artery disease, and stroke, causing morbidity and high mortality. Hypertension is known to produce pathological changes in the vasculature, such as microangiopathy in the retina, kidneys, and other organs. However, there is insufficient data on the effect on hypertension in the intestinal vasculature [13, 14, 16].

A positive correlation between trimethylamine N-oxide in plasma at birth (TMAO) and the possible increased risk of major cardiovascular adverse events has been suggested; however, the value of diagnosing TMAO levels in blood in cardiovascular diseases is questionable. However, if the nutrient concentration exceeds the transport capacity of the small intestine, they reach the large intestine and are metabolized by intestinal bacteria that produce trimethylamine (TMA). Therefore, the concentration of TMAO in the blood may depend on a few factors, including diet, intestinal microbial activity, GBB permeability to TMA, liver and TMA oxidation, and TMA and TMAO excretion [14].

Dysbiosis can contribute to the evolution of high blood pressure, another risk factor for cardiovascular disease. Dysbiosis promotes hypertension by modifying vascular tone and developing vascular fibrosis [39]. Hypertension can be defined as reducing the arterial lumen with increased peripheral vascular resistance, resulting in increased blood pressure (BP). Also, intestinal dysbiosis contributes to hypertension by vasoconstriction induced by LDL oxidation [8].

High blood pressure is a major factor contributing to an increased risk of cardiovascular disease in patients with diabetes. The presence of hypertension in patients with type 2 diabetes increases the risk of myocardial infarction, stroke, and all-cause mortality. Combining both conditions increases the risk of heart failure, nephropathy, and other microvascular events [30, 40].

There is an association between diabetes mellitus and atrial fibrillation. Both have common precursors of hypertension, atherosclerosis, and obesity. Diabetes results from defects in insulin and glucose control. This, in turn, can directly affect the atrial and ventricular myocardium. The underlying mechanism of atrial fibrillation may be linked to inflammation, with high levels of C-reactive protein and also atrial fibrosis. Diabetes is also associated with the formation of proinflammatory mediators. Left ventricular hypertrophy (LVH) is a common consequence of high blood pressure, and both are recognized risk factors for atrial fibrillation. Multiple studies have shown an association with left ventricular hypertrophy and low glucose tolerance and insulin resistance [46].

Microbial intestinal changes have been linked to changes in insulin sensitivity and in glucose metabolism and the development of metabolic syndrome with diabetes and subsequent cardiovascular complications [26]. Reduced global microbial diversity in subjects with type 2 diabetes mellitus, diminution of Firmicutes bacteria (including Clostridia), and the occurrence of proteobacteria correlate with increased plasma glucose in the oral glucose tolerance test [47]. In

diabetic patients, TMAO was found to be a significant marker for cardiovascular events. In addition, L-carnitine plasma concentrations in patients with high TMAO concentrations predicted an increased risk of cardiovascular disease and an increased incidence of major cardiac events [2, 6].

High plasma TMAO levels are associated with diastolic dysfunction and increased morbidity and mortality. TMAO may also cause ventricular remodeling by fibrosis, subsequent dilatation, thinning of the walls, and reduction of the ejection fraction [21]. Similarly, elevated levels of choline and betaine showed only an increased cardiovascular risk when associated with the concomitant increase in TMAO. These studies have enhanced the importance of dietary and antimicrobial therapy in cardiovascular health; TMAO level is a possible target for therapeutic interventions [27].

4. Treatment

Changing one's lifestyle can lead to a reduction in the risk of chronic illness, including obesity and diabetes [48].

Cardiovascular disease, the leading cause of death worldwide, poses an interest in investigation of intestinal microbiota as an interventional mechanism which yields new and clinically relevant information for future research and has a complex therapeutic potential [49].

Intervention on intestinal microbiota has already become a new target for both the prevention and treatment of complex cardiometabolic diseases [4].

The intestinal microbiota can be modulated by the administration of antibiotics, prebiotics, and probiotics or by fecal transplantation [26].

Prebiotics have protective effects and may reduce the risk of cardiovascular disease and diabetes by having a positive impact on the growth of beneficial microbial flora. Probiotics are new therapies for treating hypercholesterolemia [2, 50].

The administration of probiotics stimulates the immune response, improves lactose tolerance, has anti-inflammatory effect, and even regulates intestinal disorders caused by obesity [1, 49]. Probiotics are living nonpathogenic microorganisms that provide benefits to the host [1, 21, 43]. The intestinal microbiota may play an important role in the pathogenesis of type 2 diabetes, influencing body weight, proinflammatory activity, bile acid metabolism, insulin resistance, and intestinal hormone modulation [51].

Fecal transplantation may reduce the risk of obesity, type 2 diabetes, insulin resistance, and increased BMI [1].

Modulation of intestinal microbiota by using probiotics, prebiotics, antibiotics, and fecal transplantation may have benefits in improving glucose metabolism and insulin resistance [26]. Resveratrol (RSV) is a natural polyphenol with prebiotic benefits found mainly in grapes and berries. Furthermore, 3-dimethyl-1-butanol (DMB) is a structural analogue of choline and an inhibitor of TMA formation by inhibiting microbial enzymes [26, 51].

The interest in the study of resveratrol has started from the fact that the incidence of cardiovascular disease can be decreased by consumption of 150-300 mL/day of red wine. This has led to the extensive use of resveratrol in food supplements with doses ranging from 10-20 mg [26]. Possible mechanisms involve alteration of eicosanoid synthesis, lipid metabolism, and platelet function, as well as inflammatory response, downregulation of proinflammatory mediators, and inhibition of activated immune cells, mainly represented by neutrophils and macrophages. One of the possible mechanisms involves reducing the regulation of inflammatory response by inhibiting the synthesis and release of proinflammatory mediators, modifying the synthesis of eicosanoids and inhibiting activated immune cells [51]. Also, 3,3-dimethyl-1-butanol (DMB) is a choline analogue that inhibits TMA-lyses, a family of bacterial enzymes that transforms multiple substrates into TMA. It is active against the synthesis of TMA not only from choline but also from L-carnitine [52].

Moreover, 3,3-dimethyl-1-butanol is found in certain balsamic vinegars, red wines, and some olive and grape seed oils [46]. DMB promotes microbial taxonomy reduction associated with low plasma levels of TMA and TMAO and has also reduced colonic dietary dependence in developing atherosclerotic lesions [29].

In addition, another drug has been described a few years ago, already known to have cardioprotective clinical effects, by lowering the L-carnitine content in the body [46]. This compound, meldonium (also called Mildronate), has been shown to decrease TMAO by preventing the use of bacterial L-carnitine [52, 53].

5. Conclusions

Type 2 diabetes is a complex metabolic disease where concomitant insulin resistance and beta cell damage lead to hyperglycemia. Its proliferation is rapidly and progressively increasing due to an increase in prevalence of obesity and maintaining a western lifestyle in developing countries. Associated complications that come about at some point are related to the major causes of morbidity, mortality, and exceptional healthcare costs. Nowadays, there are no interventional clinical studies showing the beneficial effect of modulating microbiota in CVDs or diabetes.

All available clinical studies are only observational.

Cardiovascular disease (CVD) is a major health problem worldwide. Prospective studies should have demonstrated that patients with diabetes have a two- or four-fold tendency to develop heart failure and acute coronary syndromes, establishing that type 2 DM is an independent risk factor for stroke and heart disease.

In this article, we highlighted aspects relating the involvement of the microbiota and its metabolites to the pathogenesis of diabetes and cardiovascular complications. The profound understanding of the mechanisms involved will allow the early detection of diabetic patients with cardiovascular risk and the formulation of therapeutic regimens in order to reduce the risk of disease progression.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Hindawi
Journal of Diabetes Research
Volume 2018, Article ID 3407306, 12 pages
https://doi.org/10.1155/2018/3407306

Review Article

The Impact of Obesity on the Cardiovascular System

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Received 12 July 2018; Revised 12 September 2018; Accepted 27 September 2018; Published 4 November 2018

Guest Editor: Gaetano Santulli

Obesity is a growing health problem worldwide. It is associated with an increased cardiovascular risk on the one hand of obesity itself and on the other hand of associated medical conditions (hypertension, diabetes, insulin resistance, and sleep apnoea syndrome). Obesity has an important role in atherosclerosis and coronary artery disease. Obesity leads to structural and functional changes of the heart, which causes heart failure. The altered myocardial structure increases the risk of atrial fibrillation and sudden cardiac death. However, obesity also has a protective effect on the clinical outcome of underlying cardiovascular disease, the phenomenon called obesity paradox. The improved cardiac imaging techniques allow the early detection of altered structure and function of the heart in obese patients. In this review, we attempt to summarize the relationship between obesity and cardiovascular diseases and outline the underlying mechanisms. The demonstrated new techniques of cardiac diagnostic procedures allow for the early detection and treatment of subclinical medical conditions and, therefore, the prevention of cardiovascular events.

1. Introduction

Obesity has been a health problem of growing significance all over the world; its prevalence is increasing in both developed and developing countries [1]. According to WHO data, 39% of the global population above 18 years of age are overweight and of these, 13% are obese. Numerous studies have demonstrated a relationship between obesity and cardiovascular diseases (stable coronary disease, acute myocardial infarction, heart failure, cardiac arrhythmias, and sudden cardiac death). The association between obesity and hypertension, diabetes mellitus, dyslipidaemias, and sleep apnoea syndrome has also been shown to increase the incidence of cardiovascular disorders [2].

Body mass index (BMI) is used for measuring the extent of obesity; however, it gives no information on fat distribution, which is of high significance in cardiovascular risk [3]. Therefore, novel clinical measurements (e.g., abdominal circumference and the calculation of waist/hip ratio) have been introduced with the aim of characterizing central or abdominal obesity. Abdominal circumference above 102 cm in the case of men and above 88 cm in the case of women qualifies as central obesity and involves increased cardiovascular risk [4]. A waist/hip ratio above 0.9 in the case of men and above 0.85 in the case of women indicates central obesity [5].

2. The Relationship between Obesity and Atherosclerosis

In the past three decades, many details of the pathophysiological processes of obesity and atherosclerosis have been revealed. Previously, both diseases had been regarded as lipid storage disorders with triglyceride accumulation in the fat tissue and cholesterol esters in atherosclerotic plaques. Nowadays, both obesity and atherosclerosis are considered chronic inflammatory conditions, in which the activation of

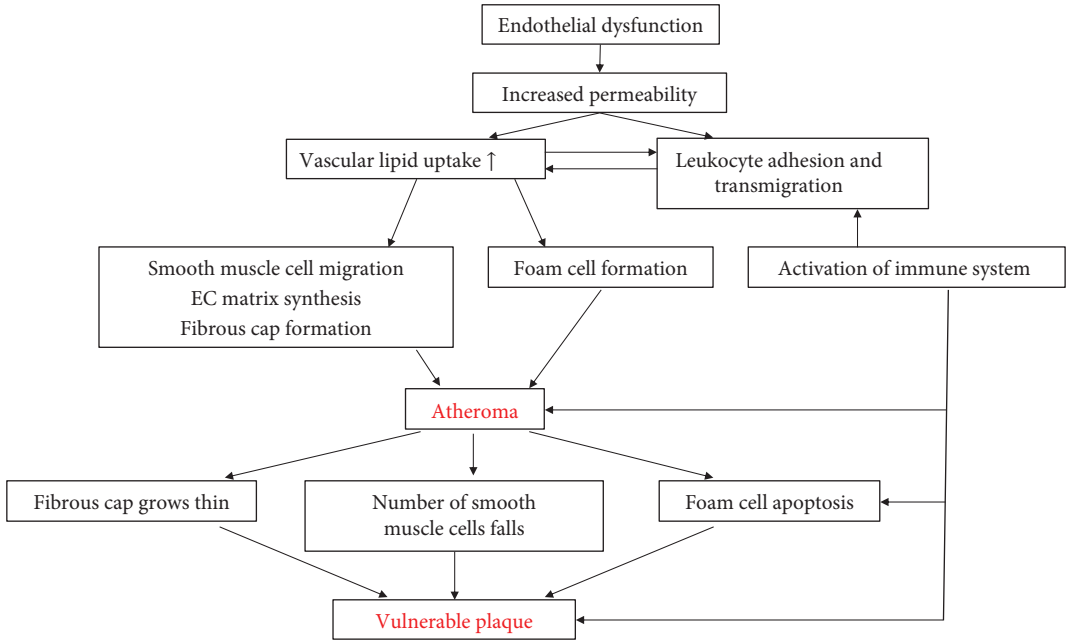

Figure 1: The pathomechanism of coronary artery disease in obesity.

both nonspecific and adaptive immune processes is assigned a significant role [6, 7].

The pathogenesis of obesity and atherosclerosis has several common factors. In both cases, lipids, oxidized LDL particles, and free fatty acids activate the inflammatory process and trigger the disease. Inflammation is responsible for all the steps towards atherosclerosis, from early endothelial dysfunction to the atherosclerotic plaques causing complications, and is related to obesity, insulin resistance, and type 2 diabetes. The fatty tissue releases adipocytokines, which induce insulin resistance, endothelial dysfunction, hypercoagulability, and systemic inflammation, thereby facilitating the atherosclerotic process. In visceral obesity, inflammatory adipocytokines (e.g., TNF-α, IL-6, MCP-1, leptin, and resistin) rise to higher levels. Moreover, the increased level of C-reactive protein is associated with an increased risk of myocardial infarction, peripheral vascular disease, and diabetes mellitus [8–10]. Interestingly, a clinical study performed on obese women confirmed that body weight reduction achieved through lifestyle changes reduces the level of inflammatory biomarkers and insulin resistance. In the course of the process, adiponectin, an anti-inflammatory and insulin-sensitizing adipocytokine, is released [11]. It is important to understand the relationship between the inflammatory process and atherosclerosis and the accelerating role of obesity.

3. Obesity and Coronary Artery Disease

Obesity is closely related to coronary atherosclerosis. A study performed on young patients showed that atherosclerosis begins several decades before manifested coronary artery disease. Atherosclerotic vascular lesions of patients with higher BMI values are more frequent and advanced compared to subjects with normal body weight [12]. According to longitudinal studies, at least two decades of obesity is likely to be an independent risk factor of coronary artery disease [13, 14]. A 10 kg rise in body weight increases the risk of coronary artery disease by 12% and at the same time, systolic blood pressure rises by 3 mmHg and diastolic by 2.3 mmHg as a consequence [15] (Figure 1). Furthermore, in the case of non-ST segment elevation myocardial infarction (NSTEMI) affecting young people, excess weight can be considered the most important risk factor, ahead of smoking. The higher the BMI, the sooner NSTEMI develops [16]. The same relationship can also be observed in the case of ST elevation myocardial infarction (STEMI) [17]. Based on the data available, obesity is an independent risk factor of STEMI developing at a young age [18] but at the same time excess weight can also be related to other vascular events. An increase in BMI by one unit causes a 4% rise in the risk of ischemic and a 6% rise in haemorrhagic strokes [19].

4. Obesity and Heart Failure

The frequency of heart failure is increasing; it is one of the major causes of death globally with a prevalence of approximately 3% in developed countries [20]. A close correlation can be observed between heart failure and obesity. According to data from the Framingham Heart Study, the rise of BMI by 1 kg/m^2 increases the risk of heart failure by 5% in the case of men and 7% in the case of women [21]. Studies on heart failure show that 32%–49% of patients suffering from heart failure are obese and 31%–40% are overweight. In the case of obese and overweight patients, heart failure develops 10 years earlier than in the case of subjects with a normal BMI. The duration of morbid obesity is closely correlated to the development of heart failure: after 20 years of obesity, the prevalence of heart failure grows by 70% and after 30 years, the

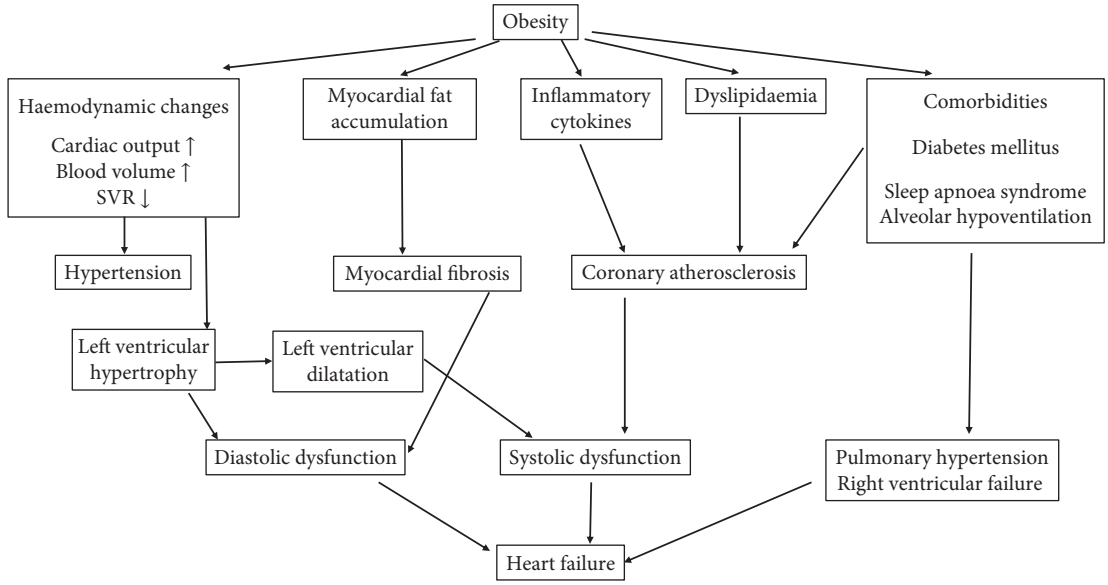

Figure 2: The pathomechanism of heart failure in obesity.

prevalence rises by 90% [22]. The significance of obesity is indicated by the fact that the Framingham Heart Study emphasized the pathogenic role of obesity for the development of heart failure in 11% of males and 14% of females [21]. The structural and functional changes of the heart observed in obesity alone contribute to a deterioration in myocardial function, which is often referred to as "obesity cardiomyopathy" [23].

Obesity leads to heart failure through several direct and indirect mechanisms. Excess weight leads to haemodynamic changes. A rise in both cardiac output and blood pressure has been observed; an increase in BMI of 5 kg/m^2 involved a 5 mmHg rise in systolic blood pressure [24]. On one hand, it is related to the activation of the renin-angiotensin-aldosterone system and on the other hand, to the increased activity of the sympathetic nervous system [25, 26]. Obesity increases both the aldosterone level and the mineralocorticoid receptor expression, which promote interstitial cardiac fibrosis, platelet aggregation, and endothelial dysfunction. The above mechanisms explain the results of EMPHASIS-HF trial: eplerenone therapy was more beneficial for treatment of heart failure with reduced ejection fraction in patients with abdominal obesity [27, 28]. Increased blood volume facilitates venous backflow, which enhances ventricular preload causing increased ventricular wall tension and ultimately leading to ventricular dilatation. Abdominal obesity is associated with subclinical left ventricular dysfunction [29]. Hypertension increases left ventricular afterload, which raises the danger of structural and electrical myocardial remodelling. This process ultimately leads to left ventricular hypertrophy and to diastolic and later to systolic ventricular dysfunction [30].

Inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8, etc.), whose production is increased in obesity, also play an important role in the development of heart failure [31–33]. The inflammatory mediators and acute-phase proteins in circulation cause myocardial fibrosis, which increases myocardial stiffness and may thereby lead to diastolic and later to systolic heart failure [34]. Through their effect on metabolism, tissue structure, and the extracellular matrix, leptin and adiponectin contribute directly to the myocardial transformation. Triglyceride accumulation in the cardiac muscle can regularly be observed in obese patients and facilitates the generation of toxic metabolites (e.g., ceramide and diacylglycerol), thus enhancing the apoptosis of cardiomyocytes [35–37]. The integrity of skeletal muscle mass is crucial for retaining the physical activity. Diet-induced obesity has been shown to promote muscle atrophy and catabolism. This process plays an important role in the progression of CVD in obese patients [38].

Moreover, obesity has been shown to increase the chances of heart failure not only by itself but also through the associated medical comorbidities. The frequently appearing insulin resistance reduces the contractility of the myocardium [39], while it enhances the activity of the renin-angiotensin-aldosterone system, which can result in hypertrophy and apoptosis of cardiac myocytes and to myocardial fibrosis [40]. Alterations in lipid metabolism enhance atherosclerosis and thereby the risk of ischemic cardiomyopathy. Unsurprisingly, obesity is therefore an independent risk factor of coronary artery disease [41]. Myocardial lipid accumulation and enhanced fibrosis can also play a pathogenic role in the genesis of various cardiac arrhythmias, which may contribute to the development of heart failure [42, 43] (Figure 2).

5. Obesity and Cardiac Arrhythmias

Numerous studies have demonstrated the connection between obesity and the increased risk of cardiac arrhythmias and sudden cardiac death [44, 45]. Hippocrates concluded in the 4th century already that "sudden death is more common in those who are naturally fat than in the lean."

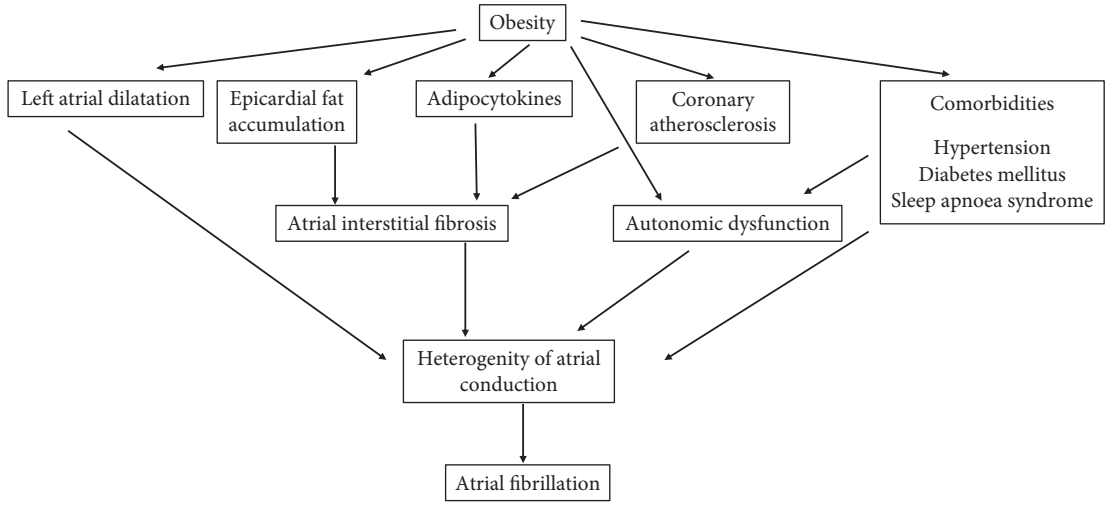

Figure 3: The pathomechanism of atrial fibrillation in obesity.

5.1. Obesity and Atrial Fibrillation. Among the cardiac arrhythmias, atrial fibrillation has the highest clinical significance. Its incidence and prevalence is increasing worldwide, affecting 1–2% of the adult population. Atrial fibrillation is responsible for about one third of the hospitalizations due to arrhythmias; it significantly increases morbidity, mortality, and health care expenditure. Over 6 million Europeans suffer from this type of arrhythmia, and this number is estimated to double in the next fifty years [46]. The occurrence of atrial fibrillation shows a correlation with age; its frequency among people aged 40–50 is under 0.5%, while it rises to 5–10% by the age of 80 years. Various studies have proven the relationship between obesity and atrial fibrillation. Obese patients have a 1.52 times higher risk for the development of atrial fibrillation compared to the normal weight population [45, 47, 48]. A 1-unit rise in BMI increases the frequency of newly developed atrial fibrillation by 4%. At the same time, in patients with atrial fibrillation, there is an increased risk for sudden cardiac death, stroke, thromboembolic complications, and heart failure. Moreover, atrial fibrillation lengthens hospitalization and worsens quality of life and physical capacity [46].

5.2. Structural and Electrical Remodelling Caused by Obesity. Obesity causes numerous anatomical and functional changes which play an important role in arrhythmogenesis. Left atrial dilation and dysfunction are known consequences of obesity. A 5 mm increase in left atrial cross diameter has been shown to raise the chances of paroxysmal atrial fibrillation 1.39 times [49]. Furthermore, recent studies have confirmed the correlation between increased epicardial fat tissue and atrial fibrillation. Through its paracrine effect, epicardial fat contributes to the development of atrial interstitial fibrosis. The increased epicardial fat, the infiltration of myocardium with adipocytes, and fibrosis together result in a heterogeneous atrial pulse conduction, e.g., anisotropy, which contributes to endo- and epicardial electrical dissociation [50–52]. All these processes facilitate the development of atrial reentry, which serves as the electrophysiological background of atrial fibrillation. Tests on both animals and humans have proven that the expansion of fatty tissue is strongly associated with insufficient capillarisation, thus myocardial hypoxia. Increased adipocyte necrosis triggers macrophage, neutrophil, and lymphocyte infiltration as well as the accumulation of proinflammatory cytokines [53] (Figure 3). It has also been proven that atrial fibrillation in obese patients shortens the refractory period of the atrial and pulmonary vein myocardial cells [54]. Enhanced adiposity triggers alterations in the ECG, too: higher amplitude P waves with lengthened duration, longer PR time, and P wave terminal force [55]. Animal research findings reveal that diet-induced obesity may be associated with prolonged atrial conduction time and heterogeneous pulse conduction. The same electrical changes were observed in the case of atrial interstitial fibrosis, increased inflammatory activity, and myocardial lipid accumulation. Remarkably, similar changes were registered in cases of congestive heart failure, hypertension, and myocardial infarction. However, the pathogenic role of complex signalisation pathways (TGF, cTGF, and endothelial system) in atrial fibrosis is not yet precisely elucidated [56].

5.3. The Role of Obesity and Low-Grade Inflammation in Arrhythmogenesis. The reason underlying atrial fibrillation is assumed to be the low-grade inflammation, which is mainly observed in relation to obesity. In obese patients, an increased number of leukocytes and a heightened presence of various inflammatory cytokines (C-reactive protein, interleukin-6, and tumor necrosis factor-α) were detected. TNF-α may increase the local arrhythmic vulnerability of the pulmonary vein, thereby causing atrial fibrillation [57, 58]. TGF-β plays an important role in the development of myocardial fibrosis [59] (Figure 3). The leptin released from adipocytes lengthens the duration of the action potential and thereby may have an arrhythmogenic effect. The roles of low-grade inflammation and oxidative stress in arrhythmogenesis have not been clearly identified yet. All the above observations

are associative, so the association through inflammation remains a hypothesis only. Modifications of the ion channel function and calcium homeostasis disorder are also assumed to be underlying phenomena [60].

5.4. Further Arrhythmogenic Factors Associated with Obesity. In the case of atrial fibrillation, the level of atrial natriuretic peptide significantly rises and shows correlation with expected mortality [61]. Additionally, in patients suffering from atrial fibrillation, the activation of the renin-angiotensin system may be associated with atrial fibrosis and electrical remodelling [62]. Furthermore, obesity may cause autonomous nervous system dysfunction. In the case of overweight patients, excessive sympathetic activity and decreased vagal tone and consequently increased urine norepinephrine secretion and cardiac rhythm disturbances were detected [63].

6. Obesity and Sudden Cardiac Death

Various studies indicate a relationship between sudden cardiac death and obesity [64]. Obesity is considered an independent risk factor in the development of ventricular tachyarrhythmias. The structural remodelling in the ventricular myocardium of obese patients results in left ventricular hypertrophy and consequential systolic and diastolic ventricular dysfunctions. Myocardial hypertrophy, fibrosis, focal myocardial disarray, and increased volume of epicardial fat are also parts of the pathological process [65].

Obesity may also be associated with prolonged and inhomogeneous ventricular repolarization, which can manifest in the prolongation of the QT interval and QT interval corrected to the heart rate (QTc) measured on the 12-lead surface electrocardiogram. These ECG parameters are known as independent markers of cardiovascular mortality, and their pathological prolongation may draw attention to an increased risk of ventricular arrhythmias [66]. In the development of the pathologically prolonged and inhomogeneous repolarization observed in obesity and the electrical instability involved as a consequence, the main roles are assigned to obesity cardiomyopathy, the altered function of voltage-dependent potassium channels, and autonomic dysregulation [67, 68].

7. The Obesity Paradox

Even though obesity involves enhanced risk for the development of cardiovascular abnormalities, in the case of an already developed disease, excess weight and obesity are associated with a favorable prognosis. The phenomenon known as obesity paradox has been observed in the case of several cardiovascular diseases including acute and chronic heart failures [69, 70], coronary artery disease [71], acute myocardial infarction [72], hypertension, and atrial fibrillation [73, 74].

7.1. The Obesity Paradox and Heart Failure. In the case of patients suffering from heart failure, the findings of numerous meta-analyses have proven the phenomenon of the obesity paradox.

According to a meta-analysis processing observational studies and summarizing the data of 28,209 patients altogether, during a follow-up time of 2.7 years on the average, in the case of overweight persons with heart failure, overall mortality was 16% lower and cardiovascular mortality was 19% lower compared to the control group. The above data are even more favorable in the case of obese patients with heart failure: overall mortality rate was 33% lower and cardiovascular mortality was 40% lower compared to normal weight patients [70].

According to a more recent meta-analysis processing the data of 22,807 patients, during an average follow-up time of 2.85 years, the relative risk of overall death in the case of overweight patients with heart failure was 0.78 (confidence interval 0.68–0.89), the relative risk of cardiovascular death was 0.79 (confidence interval 0.7–0.9), and that of hospitalization was 0.92 (confidence interval 0.86–0.97) compared to normal weight patients with heart failure. At the same time, no favorable changes were observed in the case of obese patients either in cardiovascular mortality or in hospitalization; only the risk of overall mortality was lower compared to normal weight patients [75].

The analysis of Heart Failure Registry of the Heart Failure Association of the European Society of Cardiology showed inverse relationship between all-cause and cardiovascular mortality and body surface area (BSA) levels. Hospitalizations due to heart failure were not associated with BSA [76].

7.2. The Obesity Paradox and Coronary Revascularisation. The correlation between BMI and the outcome of clinical revascularisation was first reported in 1996, in the case of patients who had been administered balloon coronary angioplasty. According to the hospital findings, the mortality rate was higher in the case of normal weight and obese patients than in that of overweight patients [77].

Data of the multicentre BARI register processing the data of 3634 patients administered coronary revascularisation (primary coronary intervention (PCI) with catheter or surgical coronary revascularisation (CABG)) reveal that in the acute hospitalization period, there was an inverse relationship between complications and BMI only in the case of PCI-treated patients. Remarkably, in the case of the CABG group, there was an inverse relationship between mortality and BMI only 5 years after the operation [78]. It must be emphasized that clinical events following surgery, like arterial hypotension, pulmonary oedema, the deterioration of kidney function, bleeding, and mortality, were more frequent in the case of thin than in that of overweight or obese patients.

According to data of the Scottish Coronary Revascularisation Register, in the case of patients who had not suffered from coronary artery disease before and who underwent elective PCI intervention, lower mortality rate was observable in the next 5 years if their BMI value was between 27 and 30 kg/m^2 [79].

According to data of the APPROACH register, mortality following PCI and CABG was more favorable in the case of overweight or obese persons compared to those with normal body weight [80].

On the contrary, other studies on coronary stent implantation did not support the phenomenon of the "obesity paradox."

Interestingly, in the course of using traditional bare metal stents (BMS), an inverse relationship could be observed between BMI and clinical outcome. In the case of BMS implantation, obesity was an independent predictor of instent restenosis. However, a relationship between obesity and adverse events was, surprisingly, not confirmed after drug-eluting stent (DES) implantation [81].

At the same time, it is still disputed if obese coronary artery disease patients would benefit from DES implantation. According to data from the German DES.DE register, the rate of hospital complications did not differ in the cases of normal weight, overweight, or obese patients. At the time of the one-year follow-up, there was similarly no difference in mortality, myocardial infarction, target vessel revascularisation, or bleeding complications [82].

7.3. Explanation for the Obesity Paradox. It is well known that excess weight and obesity, as phenomena of the metabolic syndrome, lead to enhanced cardiovascular risk, endothelial dysfunction, inflammation, and atherosclerosis. The most important question is what can the explanation be for the better prognosis established in the case of overweight and obese cardiovascular patients compared to normal weight patients.

The analyses show that in the case of 2% of thin patients, comorbid conditions, mostly malignant diseases, heart failure, malnutrition, or multiple organ dysfunction, can be observed. Moreover, these patients were much older than their normal weight or obese counterparts [78, 83]. Obviously, in the case of older age patients in a generally weak condition, clinical outcomes after coronary events proved to be worse irrespective of the success of the reperfusion [84]. Advanced age and comorbid factors often result in loss of body weight [85]. In obesity, the increased level of serum lipoproteins may neutralize bacterial toxins and circulating cytokines [86]. The low level of adiponectin and the reduced catecholamine response, too, may increase the chances of survival [87]. Furthermore, in the case of obese patients, cardiovascular diseases are usually diagnosed and treated earlier than in the case of thin patients [88]. In the case of overweight and obese patients, the dose of medication required in treating the cardiovascular disease is easier to titrate considering the associated hypertension and obese patients are also more compliant with regimen than their normal weight counterparts. A possible explanation of obesity paradox is that in critical ill patients, fat which mobilized from excess adipose tissue provides energy and prevents lean tissue wasting more efficiently than exogenous nutrients [89]. In heart failure, a metabolic cardiac remodeling occurs, the fatty acid oxidation is impaired, and the glucose uptake and glycolysis are increased. The metabolic imbalance between higher energetic demand and substrate availability and lower oxidative capacity and cofactor availability (carnitine and CoA) leads to the accumulation of intermediates, which impair cardiac function, and substrates are diverged towards lipotoxic signaling pathways [90]. Alterations in mitochondrial dynamics, respiratory capacity, and ATP synthesis play an important role in the chronic cardiac energy deficit observed in heart failure [91]. Improved fatty acid utilization via dietary modification significantly ameliorates mitochondrial fragmentation and cardiac dysfunction [92].

According to more recent theories explaining the "obesity paradox," obese patients have "larger blood vessels" and in the course of PCI, worse results are obtained in the case of patients with narrowed blood vessels [93, 94].

Antithrombotic medication is usually administered in standard doses rather than adjusted to body weight, so the dose may be too high for normal weight and thin patients, which may result in bleeding complications and this, in turn, may also contribute to higher mortality [95].

If the outcomes of cardiopulmonary stress tests are also considered, the favorable effect of higher BMI was shown to disappear [96]. According to other views, the greater muscle strength associated with a higher BMI has a favorable effect on so-called "cardiorespiratory fitness" [97, 98]. Peak oxygen consumption (VO_2) is a positive predictor of longer survival among patients with heart failure. In multivariate analysis using VO_2, the protective role of BMI for survival disappears [99]. The survival paradox of BMI disappears also in diabetic patients with heart failure [100]. These results support the superior prognostic power of peak oxygen consumption and diabetes compared to obesity, which attenuates the "obesity paradox" phenomenon [101].

According to the endotoxin-lipoprotein hypothesis, obese patients have higher cholesterol and lipoprotein levels, which reduce the concentration of inflammatory agents and may thus have anti-inflammatory and probably also arrhythmia protective effect. The observation that the myocardial accumulation of fat enhances the density of TNF-α I and II receptors, thereby facilitating the development of an antiarrhythmogenic environment, may at the same time probably serve as a kind of explanation for the development of the obesity paradox [57, 58].

8. Cardiac Consequences of the Haemodynamic Changes Triggered by Obesity

The volume stress associated with obesity causes both structural and functional changes in the heart. The most frequent structural changes are left ventricular hypertrophy (LVH) and left ventricular dilation, and as a consequence, diastolic, then systolic dysfunction, epicardial fat accumulation, and left atrial enlargement occur. The circulating blood volume rises; the increased cardiac output is provided mainly by the increased stroke volume and, to a lesser extent, by the increased cardiac frequency as an effect of the enhanced sympathetic tone. The above mechanisms often cause hypertension [23] (Figure 2).

9. Cardiology Diagnostics in Obesity

Considering the enhanced cardiovascular risk and inclination to arrhythmia observed in obesity, cardiology diagnostics are important even in the case of symptom-free obese patients. The routine 12-lead surface ECG and

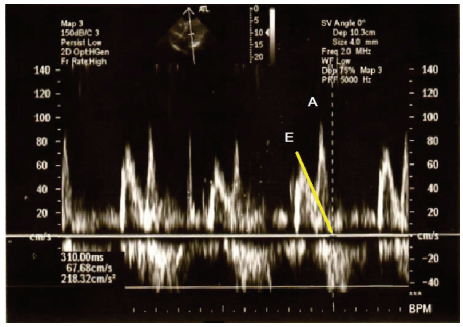

Figure 4: Diagnosing diastolic dysfunction with echocardiography. Transmitral flow velocities measured with pulsatile wave Doppler technique. The ratio (E/A) of the early diastolic peak velocity (E) and the late diastolic velocity (A) is lower than 1. Deceleration time (DT) is the interval from the peak of the wave E to its end (marked with a yellow line). In this case, its prolongation was measured (310 sec). The above alterations prove the left ventricular diastolic dysfunction (relaxation disorder).

echocardiography are available at almost any cardiology outpatient unit nowadays.

9.1. Echocardiography. According to a meta-analysis published in 2014, the frequency of left ventricular hypertrophy (LVH) in obese patients is 56%; the risk of the development of LVH in the case of obese patients is 4.19 times higher than in the case of people with normal weight. Excentric hypertrophy is more frequent than the concentric type (66% vs. 34%, $p < 0.001$) [102]. LVH is usually calculated by the left ventricular mass index (LVMI), whereby it is indexed to height$^{2.7}$. This shows a good correlation with cardiovascular mortality. Less frequently, indexation to body surface area is used [103].

In obesity, the prevalence of diastolic dysfunction is above 50% and shows close correlation with abdominal circumference. Among cardiovascular risk factors, age, sex, and hypertension increase the likelihood of diastolic dysfunction, which is demonstrated by what is referred to as the E/A ratio: the ratio of the mitral flow velocities measured in the early diastole (E) and late diastole (A). The value of the ratio is less than 1 in the case of diastolic dysfunction, primarily due to a rise in the peak velocity of A. The diagnosis also requires establishing the so-called deceleration time, i.e., the time taken from the peak to the end of the A wave, while the velocities of the mitral annular longitudinal movement obtained by the tissue Doppler imaging technique constitute further complementary and specification data (Figure 4). Left atrial volume enlargement is also often associated with diastolic dysfunction and can thus be considered as a marker of the latter [104]. At the same time, conventional echocardiography is sometimes unsuitable for the early diagnosis of systolic or diastolic dysfunction as the measurable parameters may still be in the normal range.

In the past decade, new echocardiographic techniques have become available that make a yet earlier diagnosis of systolic and diastolic dysfunctions possible [105]. Color Doppler imaging detects the movement and deformity of the myocardium and is thereby able to show changes in contractility [106]. The so-called "integrated backscatter" technique is able to sense changes in the reflectivity and weakening of the myocardium. These are primarily determined by the myocardial collagen content and are also influenced by the size and microstructure of cardiac muscle cells. The technique primarily provides information on myocardial stiffness, contractility, and the extent of fibrosis, in a noninvasive way [107, 108]. Pulsatile wave tissue Doppler imaging (PW-TDI) measures the cardiac muscle movement velocity. These Doppler parameters are more precise and easier to reproduce than those obtained by means of 2D, M-mode echocardiography [109]. 3D imaging has lately been introduced in cardiology as well, which makes the determination of the ejection fraction (EF) and the volume of the left atrium and the left ventricle more precise [110]. A comparison with MRI imaging proved the advantages of 3D echocardiography.

9.2. Electrocardiography. In case of obesity, the QT interval corrected to the heart rate (QTc) is prolonged and QT dispersion (QTd) also increases [111, 112]. These electrocardiographic differences show a correlation with an enhanced disposition to ventricular arrhythmia. In the past two decades, new markers of ventricular repolarization have been identified, which characterize cardiac muscle vulnerability in coronary artery disease, hypertrophic cardiomyopathy, and long QT syndrome very well: T peak-end (Tpe) interval, T peak-end dispersion, and Tpe/QT ratio (arrhythmogenic index) [113–115]. Studies performed on obese patients showed, however, that statistically significant prolongation compared to the control values was observed only in the case of QT interval and QTc; there were no similar differences observed in the case of the other electrocardiographic parameters [116].

Using the above testing procedures, the slight structural, electrical, and functional changes of the heart can be detected. Consequently, symptom-free patients with enhanced risk for ventricular arrhythmias can be identified at an early stage.

10. Summary

Excess weight and obesity are associated with an increased risk of cardiovascular diseases. This is a consequence on the one hand of obesity itself and on the other hand of associated medical conditions (hypertension, diabetes, insulin resistance, and sleep apnoea syndrome). In case of already established cardiovascular diseases, the mortality of overweight and obese patients is often lower than that of people with a normal body weight, which is known as "obesity paradox." The exact mechanism of the latter is not clear yet. Considering the increased cardiovascular risk, the regular cardiology screening, and control of still symptom-free obese patients is important for the early diagnosis and treatment of subclinical medical conditions.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This research was carried out with support from the GINOP-2.3.2-15-2016-00005 fund.

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Hindawi
Journal of Diabetes Research
Volume 2018, Article ID 4603087, 7 pages
https://doi.org/10.1155/2018/4603087

Research Article

The U-Shaped Association between Bilirubin and Diabetic Retinopathy Risk: A Five-Year Cohort Based on 5323 Male Diabetic Patients

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Received 14 May 2018; Revised 23 August 2018; Accepted 11 September 2018; Published 30 October 2018

Guest Editor: Markus Wallner

Aims. This study aimed at assessing the impact of baseline bilirubin (TBiL) on the incidence of diabetic retinopathy (DR) based on a five-year cohort study which consisted of 5323 Chinese male diabetic patients. *Methods*. A cohort study based on 5323 male diabetic patients was conducted in Beijing, from 2009 to 2013. Both baseline TBiL and follow-up changes were measured. Cox proportional risk model was used to calculate the hazard ratio (HR) of TBiL for DR risk. *Results*. During the follow-up period, there were 269 new DR cases. The incidence of five-year follow-up was 5.1% (95% CI: 4.5%~5.6%). The TBiL level of those who had diabetic retinopathy was lower than that of those without (12.51+ 1.20 mol/L and 13.11+ 1.32 μmol/L, $P = 0.033$). And more interestingly, along with the quintiles of baseline TBiL, there showed a U-shaped curve with DR incidence. And the RRs were 0.928 (95% CI: 0.646–1.331), 0.544 (95% CI: 0.365–0.811), 0.913 (95% CI: 0.629–1.324), and 1.035 (95% CI: 0.725–1.479) for the second, third, fourth, and fifth quintiles of baseline TBiL levels, respectively, compared with the first quintile. For follow-up TBiL changes, after being adjusted for related covariables and baseline TBiL levels (as continuous variable) in the model, the RRs for DR were 1.411 (95% CI: 1.081–1.842) for those who had decreased TBiL level and 0.858 (95% CI: 0.770–0.947) for those who had increased TBiL level during follow-up. And this association was more prominent among those with lower baseline TBiL level. *Conclusions*. Serum TBiL had a U-shaped relationship with DR incidence, which was independent of control status of diabetes and other related covariates.

1. Introduction

Diabetic retinopathy (DR) is one of the important vascular complications of diabetes. Data show that this is the main cause of blindness among working age population in developing countries [1]. Therefore, exploring the pathogenesis of DR is of most importance [2]. Previous basic and clinical research data indicated that oxidative stress played an important role in the development of DR.

Total bilirubin (TBIL) has been considered as a powerful endogenous antioxidant in recent years. A number of studies have shown that elevated TBiL levels were negatively correlated with cardiovascular disease and diabetes mellitus [3–5]. In addition, a number of studies have reported a protective relationship between TBiL levels and diabetic vascular complications. There was also a meta-analysis of the association between TBiL and DR published in 2016 [6]. However, these studies were mostly cross-sectional or case-control studies with small sample sizes and inconsistent findings. Some studies show that there was no association between TBiL and DR risk [7, 8]. In addition, there was little evidence of dose-response effects, which was of great value in determining appropriate clinical thresholds of TBiL levels among diabetic patients. Therefore, based on this five-year

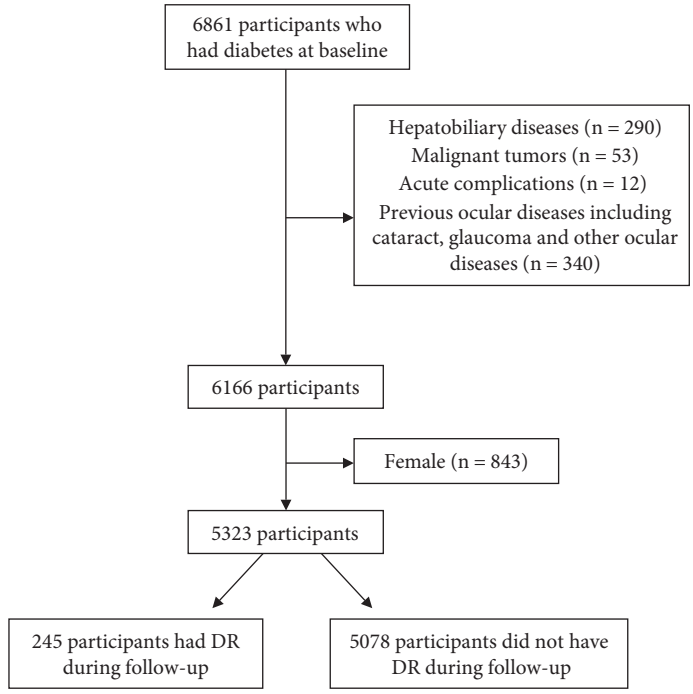

Figure 1: Flow chart of inclusion of participants.

cohort study with a large sample (more than 5000 elderly diabetic patients), our study assessed the relationship between TBIL and its changes and the incidence of DR.

2. Methods

2.1. Subjects. This cohort study consisted of elderly diabetic patients. We conducted the baseline survey in 2009, and a total of 6861 elderly were recruited, and the follow-up survey was done in 2013. The details were described in a previous article [9]. Considering the possible effects of related diseases on TBiL or diabetes, we excluded those patients who had hepatobiliary diseases ($n = 290$), malignant tumors ($n = 53$), acute diabetic complications such as ketoacidosis ($n = 12$), previous ocular diseases including cataract, glaucoma, and other ocular diseases ($n = 340$). Considering female just accounted for 13.7% (843) of the total participants, only 5323 male participants were left for further analysis. Figure 1 shows the flow chart of participants' inclusion.

2.2. Data Collection. Baseline anthropometric and physical examination information was collected according to the standard process. Fasting blood was collected, and the related biochemical indexes were detected. The details were described in a previous article [9].

2.3. Definitions. The duration of diabetes was calculated by age minus the age of first diagnosis on diabetes. Quintiles of baseline TBiL level were defined as follows: Q1: ≤9.20 μmol/L, Q2: 9.20–12.60 μmol/L, Q3: 12.60–13.80 μmol/L, Q4: 13.80–16.50 μmol/L, and Q5: ≥16.50 μmol/L. Follow-up changes of TBiL were defined as follow-up TBiL levels minus baseline TBiL levels and were divided into three categories: ≤−2 μmol/L; −2 to 2 μmol/L; and ≥2 μmol/L. Diabetes, hypertension, and dyslipidemia were defined according to the corresponding guidelines. DR was defined according to the Chinese version of guidelines for the prevention and treatment of type 2 diabetes: the presence of mild or moderate or proliferative retinopathy in either eye [10], and was independently diagnosed by two senior ophthalmologists.

2.4. Statistical Analysis. For continuous data, mean ± SD was used for description, and analysis of variance was used for comparisons. For categorical variables, chi-square test is used for comparison. Multivariate Cox proportional hazard model was used to estimate the hazard ratio (HR) and 95% confidence intervals (CIs) of DR based on baseline TBiL levels and follow-up TBiL changes. Restricted cubic spline functions with 5 knots were used to test the potential nonlinear association and display the data graphically. SPSS software was used for data analysis. $P < 0.05$ was statistically significant.

2.5. Ethical Consideration. The ethics committee of the General Hospital of PLA approved the study (EC0411-2001). Each participant signed a written informed consent.

3. Results

3.1. Baseline Characteristics according to DR Incidence. Table 1 shows the baseline characteristics of the subjects. The mean age of the 5323 diabetic patients was 78.68 ± 8.39 (65~102 yrs). Mean diabetes duration and TBiL level were 17.25 ± 7.49 yrs and 13.08 ± 1.32 μmol/L. The percentage of overweight/obesity, hypertension, and dyslipidemia was 68.4%, 62.4%, and 35.8%, respectively. Compared with those who did not have DR, those with DR had relatively longer duration, higher FPG and 2hPG levels, and lower

Table 1: General characteristics of participants according to DR incidence.

Characteristics	NDR ($n = 5078$)	DR ($n = 245$)	P	Total ($n = 5323$)
Mean ± SD				
Age (yrs)	78.68 ± 8.41	78.95 ± 7.80	0.623	78.68 ± 8.39
Duration (yrs)	17.14 ± 7.51	19.81 ± 7.40	0.042	17.25 ± 7.49
Height (cm)	169.64 ± 5.41	169.22 ± 5.18	0.235	169.62 ± 5.40
Weight (kg)	72.61 ± 8.53	72.66 ± 8.51	0.927	72.61 ± 8.53
BMI (kg/m^2)	25.24 ± 2.79	25.38 ± 2.80	0.446	25.24 ± 2.79
SBP (mmHg)	133.28 ± 14.04	133.77 ± 13.64	0.593	133.30 ± 14.02
DBP (mmHg)	73.88 ± 9.46	72.68 ± 8.97	0.052	73.82 ± 9.44
Hb (g/L)	132.11 ± 15.44	131.16 ± 20.37	0.848	132.07 ± 17.83
TC (mmol/l)	4.75 ± 1.05	4.68 ± 1.00	0.307	4.75 ± 1.05
TG (mmol/l)	1.68 ± 1.08	1.59 ± 1.00	0.196	1.67 ± 1.07
HDL-C (mmol/l)	1.33 ± 0.44	1.23 ± 0.32	<0.001	1.33 ± 0.44
LDL-C (mmol/l)	2.67 ± 0.83	2.74 ± 0.83	0.186	2.67 ± 0.83
FPG (mmol/l)	6.93 ± 1.89	6.95 ± 1.92	0.046	6.94 ± 1.90
2hPG (mmol/l)	9.24 ± 2.83	9.96 ± 2.35	<0.001	9.27 ± 2.86
ALT (U/L)	20.81 ± 4.59	19.91 ± 4.99	0.041	20.76 ± 4.68
Baseline TBiL (μmol/L)	13.11 ± 1.32	12.51 ± 1.20	0.033	13.08 ± 1.32
%				
Education			0.105	
≤6 yrs	65.1	65.3		65.4
≥7 yrs	34.9	34.7		34.6
Marriage status			0.077	
Divorced/widowed	16.5	12.3		12.7
Married	87.5	83.7		87.3
Current smoking			0.186	
Yes	21.7	18.6		21.5
No	78.3	81.4		78.5
Current alcohol drinking			0.854	
Yes	19.7	19.5		18.7
No	80.3	80.5		80.3
Overweight/obesity			0.640	
Yes	68.4	69.8		68.4
No	31.6	30.2		31.6
Hypertension			0.171	
Yes	62.2	66.5		62.4
No	37.8	33.5		37.6
Dyslipidemia			0.101	
Yes	34.8	35.3		35.8
No	65.2	64.7		64.2
Control of diabetes			0.907	
Yes	50.7	50.5		50.8
No	49.3	49.5		49.2

Data are mean ± SD for continuous values or % for category values.

baseline TBiL level ($p < 0.05$). Baseline characteristics according to quintiles of baseline TBiL levels were presented in Appendix Table 1. Along with the increase of baseline TBiL levels, it showed shorter duration; higher Hb, HDL-C, FPG, and 2hPG levels; and lower percentage of diabetes control status.

3.2. Incidence of DR according to Baseline TBiL Quintiles and Follow-Up TBiL Changes. There were a total of 269 DR cases during the 21,586 person-years. The total five years' incidence was 5.1% (95% CI: 4.5%–5.6%). As we can see from Table 2, the incidence of the quintiles of baseline TBiL for DR had fluctuations; the third quintile

Table 2: Incidence of DR according to baseline TBiL quintiles and follow-up TBiL changes.

DR	Quintiles of baseline TBiL(μmol/L)					Follow-up TBiL changes (μmol/L)			Total
	Q1 (≤9.20)	Q2 (9.20–12.60)	Q3 (12.60–13.80)	Q4 (13.80–16.50)	Q5 (≥16.50)	≤−2	−2 to 2	≥2	
Number of incident cases	61	57	40	51	60	115	105	49	269
Incidence (%)	5.6 (4.3–7.0)	5.4 (4.0–6.8)	3.4 (2.4–4.5)	5.2 (3.8–6.2)	5.7 (4.3–7.1)	6.3 (5.2–7.5)	4.9 (4.0–5.9)	3.7 (2.7–4.8)	5.1 (4.5–5.6)
Total person-years	4399	4302	4638	3981	4267	7443	8692	5451	21,586
Incidence density (per 100 person-years)	1.4 (1.1–1.8)	1.3 (1.0-1.7)	0.9 (0.6–1.2)	1.3 (1.0-1.7)	1.4 (1.1–1.8)	1.5 (1.3–1.9)	1.2 (1.0-1.5)	0.9 (0.7–1.2)	1.2 (1.1–1.4)

Table 3: HRs and 95% CI of DR incidence according to baseline TBiL levels (μmol/L).

Variable type	HR* (95% CI)	*P*
Continuous variable	0.956 (0.934–0.983)	0.001
Quintiles		0.017
Q1 (≤9.20)	1.838 (1.233–2.740)	
Q2 (9.20–12.60)	1.705 (1.138–2.555)	
Q3 (12.60–13.80)	1.00 (Ref)	
Q4 (13.80–16.50)	1.678 (1.109–2.540)	
Q5 (≥16.50)	1.903 (1.275–2.841)	

*Adjusted for age, marital status, current smoking, current alcohol drinking, BMI, baseline Hb, ALT, baseline prevalence of hypertension and dyslipidemia, control of diabetes, duration of diabetes, and follow-up TBiL changes (as continuous variable) in the model.

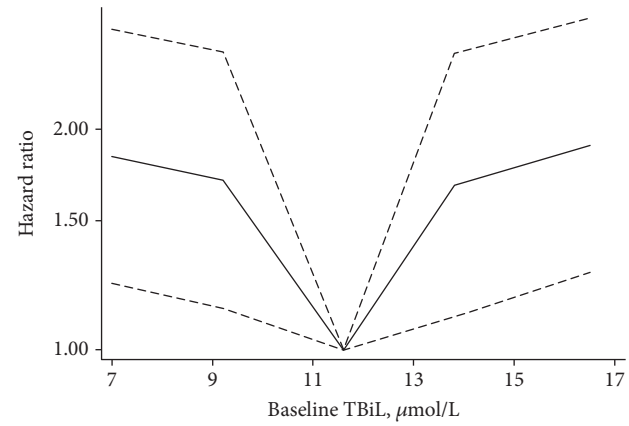

Figure 2: HRs of follow-up TBiL changes for DR incidence according to baseline TBiL level.

had the lowest incidence while the first and the fifth had the highest incidence. The incidence density showed a similar trend. The incidence of follow-up TBiL changes for DR was lowest among those who had an increase of ≥2 μmol/L while highest among those who had a decrease of ≤−2 μmol/L (Table 2).

3.3. HRs and 95% CI of DR Incidence according to Baseline TBiL Levels. Table 3 showed the HRs of baseline TBiL levels for DR incidence. We adjusted related covariables according to results of univariate analysis (Appendix Table 1); the HRs of baseline TBiL levels for DR were 0.9656 (95% CI: 0.934–0.983). When quintiles were used as categorical variables, the HRs were 1.838 (95% CI: 1.233–2.740), 1.705 (95% CI: 1.138–2.555), 1.678 (95% CI: 1.109–2.540), and 1.903 (95% CI: 1.275–2.841) for the first, second, fourth, and fifth quintiles of baseline TBiL levels, respectively, compared with the third quintile. And restricted cubic spline functions depicted a sort of U-shaped curve (Figure 2).

3.4. HRs for DR Incidence according to Follow-Up TBiL Changes. The follow-up TBiL level was lower than the baseline TBiL level. The mean level of the baseline TBiL was 13.05 ± 1.65 (median: 12.30, IQR: 9.20–15.30) μmol/L, and the mean follow-up TBiL level was 12.54 ± 1.64 (median: 12.50, IQR: 10.00–15.70) μmol/L; thereby, the follow-up TBiL changes were −0.66 ± 1.93 (median: −0.73, IQR: −3.30 to 2.10) μmol/L (Appendix Table 2). Those who had DR in the follow-up years had a relatively bigger TBiL change; the mean follow-up TBiL changes were − 1.60 ± 1.48 (median: −1.11, IQR: −3.86 to 1.20) μmol/L.

For follow-up TBiL changes, after being adjusted for related covariables and baseline TBiL levels in the model, the HRs for DR were 0.967 (95% CI: 0.944–0.991). When used as categorical variables, as we can see from Table 4, compared with those who had relatively stable TBiL levels (−2 μmol/L < follow-up TBiL changes < 2 μmol/L), the HR for DR incidence was higher (HR = 1.411, 95% CI: 1.081–1.842) among those who had follow-up TBiL changes ≥2 μmol/L, and the HR was lower (HR = 0.858, 95% CI: 0.770–0.947) among those with follow-up TBiL changes ≤−2 μmol/L. Besides, the decreasing trend of follow-up TBiL changes for DR incidence was more obvious among those with lower baseline TBiL level (≤12.5 μmol/L, $n = 2669$). However, among those with relatively higher baseline TBiL level (>12.5 μmol/L, $n = 2654$), the HRs of follow-up TBiL changes showed no significant results.

In the sensitivity analysis, when participants who had DR that happened within less than 2 years were excluded ($n = 37$) or divided by age groups (≤80 yrs and >80 yrs), the trend of adjusted HRs was similar with the results from Tables 3 and 4 (Appendix Tables 3, 4, 5, and 6).

Table 4: HRs and 95% CI of DR incidence according to follow-up TBiL changes (μmol/L).

	Variable type	HR* (95% CI)	P
Total population ($n = 5323$)	Continuous variable	0.967 (0.944–0.991)	0.006
	Categorical variable		0.011
	≤−2	1.411 (1.081–1.842)	
	−2 to 2	1.00 (Ref)	
	≥2	0.858 (0.770–0.947)	
Among those with baseline TBiL level ≤ 12.5 μmol/L ($n = 2669$)	Continuous variable	0.964 (0.931–0.986)	0.037
	Categorical variable		0.003
	≤−2	1.756 (1.217–2.534)	
	−2 to 2	1.00 (Ref)	
	≥2	0.853 (0.761–0.934)	
Among those with baseline TBiL level > 12.5 μmol/L ($n = 2654$)	Continuous variable	0.969 (0.936–1.003)	0.072
	Categorical variable		0.794
	≤−2	1.059 (0.689–1.626)	
	−2 to 2	1.00 (Ref)	
	≥2	0.864 (0.451–1.654)	

*Adjusted for age, marital status, current smoking, current alcohol drinking, BMI, baseline Hb, ALT, baseline prevalence of hypertension and dyslipidemia, control of diabetes, duration of diabetes, and baseline TBiL levels (as continuous variable) in the model.

4. Discussion

In this study, we did a deep study and evaluated the relationship between baseline TBiL and DR incidence using a large cohort of more than 5000 male elderly. The results showed that baseline TBiL had a U-shaped relationship with DR incidence, rather than a simple linear relationship. And this association was independent of control status of diabetes and other related covariates. And more interestingly, increased follow-up TBiL changes had a higher DR risk, and this association was more prominent among those with lower baseline TBiL level.

There were several researches focused on relationships between TBiL level and DR risk [11, 12], even one meta-analysis published in 2016 [6]. Most of the previous studies showed that there was a negative relationship between TBiL and DR. However, the result of our deep study showed that the association between TBiL and DR risk was not a simple linear association but a U-shaped curve. From the meta-analysis of TBiL and DR, we could see that most studies were cross-sectional ones or case-control studies with a small sample. Our study was based on a five-year cohort which consisted of more than 5000 elderly diabetic patients. And this U-shaped curve was consistent with previous ones about relationships about TBiL with cardiovascular diseases. A cohort study based on 7685 middle-aged British men firstly revealed that there was a U-shaped relationship between TBiL and risk of ischemic heart disease [13]. And in 2012, results from one of the biggest cohorts based on more than 130,000 patients who received statin treatment showed that a U-shaped association appeared between TBiL before statin prescription and coronary heart diseases [14]. Our result was consistent with these prospective studies [15]. And this implies that the DR risk would no longer decrease when baseline TBiL increased to a relatively higher level. Meanwhile, there were a series of evidences showing that a higher TBiL level (beyond normal range) indicated hepatocellular injuries, and the latter one was proved to be associated with increased risk of diabetes and cardiovascular disease [7]. Besides, the negative relationship might be part of the U-shaped curve, since most of the cross-sectional studies were of small sample size. And the actual U-shaped association might be the combination of antioxidation and liver toxicity effects.

The pathogenesis of DR has not been fully studied. Oxidative stress caused by high glucose is one of the hotspots of the current research [16]. TBiL, not only a metabolite of hemoglobin, is also considered to be an important endogenous antioxidant. Past studies have shown that TBiL has important protective effects on cardiovascular disease, diabetes, and diabetic macrovascular complications, mainly through anti-inflammatory and antioxidant effects [17, 18]. For DR, studies have revealed that TBiL has significant protective effects [6]. And results from the first known human case of heme oxygenase-1 deficiency showed that TBiL at physiological concentration has strong antioxidant activity, which can prevent low-density lipoprotein lipid peroxidation, and results based on mice showed that 1 mol/L direct bilirubin can remove about 2 mol/L free radicals [19]. All these evidences support the protective effect of TBiL on DR. However, the premise is in the normal range. If the TBiL was beyond the normal range, it is most probably an indicator of liver damage, which was an important risk factor for diabetes and its complications. There were several prospective studies with large sample size including our study which have showed the protective effect of TBiL in the normal range and the harmful effect in the relative higher range for cardiovascular disease and diabetes (including its complications) [14, 15, 20], which confirmed the U-shaped curve of TBiL for DR in our study. This implies that in clinical practice, the previous "higher TBiL indicates lower risk of complications" was not always right. Doctors should also pay enough attention to the abnormal range and whether there were liver toxicity effects.

Follow-up changes of TBiL also had protective effects on DR incidence, which indicated that medical workers should also pay attention to both the baseline and the fluctuation of TBiL levels among diabetic patients, and it will play an important role in predicting DR incidence. Most of the previous studies only assessed the relationship between baseline TBiL level and DR; however, only a few studies focused on

the follow-up volatility. In our study, for those who had increased TBiL changes (≥2 μmol/L), the risk of DR incidence had increased about 40%. And the more obvious decreasing trend among those with relatively lower baseline TBiL levels verified previous results about the U-shaped curve of the association between TBiL and DR risk.

As far as we know, this was the first study to analyze the relationship between TBiL and DR based on a large sample cohort in fully adjusted models. In addition, besides baseline TBiL levels, TBiL changes also had an independent and inverse impact on DR incidence. The cohort had rigorous investigation process, strict training for all the staff in the field survey, and high response rate because of the well-controlled follow-up system.

However, this study had the following limitations. First, TBiL was measured only once at either baseline or follow-up, so it could not reflect the actual fluctuations. Second, only TBiL was collected; there was no specific value for direct and indirect bilirubin levels, so it was impossible to distinguish the exact role of these two types. Third, all participants were retired male, whose economic and medical security was relatively good, and the representation was limited for the general population.

In summary, this large-sample cohort study had shown that TBiL and its changes in diabetic patients were independently associated with DR incidence, and this association was independent of both classical risk factors and diabetes control status. Clinical medical staff should pay attention to the monitoring of TBiL levels in order to early detect and better control DR.

Abbreviations

ALT: Alanine aminotransferase
BMI: Body mass index
CI: Confidence interval
DBP: Diastolic blood pressure
DR: Diabetic retinopathy
FBG: Fasting plasma glucose;
HDL-C: High-density lipoprotein cholesterol
HR: Hazard ratio
LDL-C: Low-density lipoprotein cholesterol
SBP: Systolic blood pressure
SD: Standard deviation
TBiL: Total bilirubin
TC: Total cholesterol
TG: Triglyceride
2hPG: Two-hour plasma glucose.

Data Availability

Data was available on reasonable request and can be obtained from the corresponding author.

Disclosure

The views and opinions expressed in this paper are those of the authors and do not necessarily reflect the official position of the study sponsors.

Conflicts of Interest

The authors declare that they have no competing interest.

Authors' Contributions

M. L. and Y. H. contributed to the design of the study. All authors were involved in the analysis and interpretation of the data. J. H. W. and M. L. conducted the statistical analysis. M. L. and Y. H. worked on the drafting of the manuscript, which was thoroughly reviewed and approved by all the authors. Miao Liu and Jianhua Wang contributed equally to this work.

Acknowledgments

We thank Dr. Bin Jiang, Dr. Jiaqi Li, Dr. Xianyu Lv, and head nurse Yafeng Yan for the help in the field survey. This study is supported by research grants from the National Natural Science Foundation of China (81773502, 81703285), Beijing Natural Science Foundation (7174350), Beijing Municipal Science and Technology Commission (Z161100005016021), Beijing Nova Program (Z181100006218085), Opening Foundation of State Key Laboratory of Kidney Diseases (KF-01-115), and Military Fund (15BJZ41, 17BJZ51).

Supplementary Materials

Appendix Table 1: the general characteristics of participants according to the quintiles of baseline total bilirubin (TBiL) levels. Appendix Table 2: the distribution (including mean, SD, median, and interquartile range) of baseline, follow-up, and change of TBiL levels (μmol/L). Appendix Table 3: the HRs and 95% CI of diabetic retinopathy (DR) incidence according to baseline TBiL levels (μmol/L) (excluding DR cases in the first two years) using the Cox model. Appendix Table 4: the HRs and 95% CI of DR incidence according to follow-up TBiL changes (μmol/L) (excluding DR cases in the first two years) using the Cox model. Appendix Table 5: the HRs and 95% CI of DR incidence according to baseline TBiL levels (μmol/L) (by different age groups, ≤80 yrs vs. >80 yrs) using the Cox model. Appendix Table 6: HRs and 95% CI of DR incidence according to follow-up TBiL changes (μmol/L) (by different age groups, ≤80 yrs vs. >80 yrs) using the Cox model. *(Supplementary Materials)*

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Hindawi
Journal of Diabetes Research
Volume 2018, Article ID 3106056, 8 pages
https://doi.org/10.1155/2018/3106056

Review Article

Role of Tight Glycemic Control during Acute Coronary Syndrome on CV Outcome in Type 2 Diabetes

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Received 15 April 2018; Revised 22 August 2018; Accepted 13 September 2018; Published 4 October 2018

Guest Editor: Markus Wallner

Both incidence and mortality of acute coronary syndrome (ACS) among diabetic patients are much higher than those among nondiabetics. Actually, there are many studies that addressed glycemic control and CV risk, whilst the literature on the role of tight glycemic control during ACS is currently poor. Therefore, in this review, we critically discussed the studies that investigated this specific topic. Hyperglycemia is implicated in vascular damage and cardiac myocyte death through different molecular mechanisms as advanced glycation end products, protein kinase C, polyol pathway flux, and the hexosamine pathway. Moreover, high FFA concentrations may be toxic in acute ischemic myocardium due to several mechanisms, thus leading to endothelial dysfunction. A reduction in free fatty acid plasma levels and an increased availability of glucose can be achieved by using a glucose-insulin-potassium infusion (GIKi) during AMI. The GIKi is associated with an improvement of either long-term prognosis or left ventricular mechanical performance. DIGAMI studies suggested blood glucose level as a significant and independent mortality predictor among diabetic patients with recent ACS, enhancing the important role of glucose control in their management. Several mechanisms supporting the protective role of tight glycemic control during ACS, as well as position statements of Scientific Societies, were highlighted.

1. Introduction

Diabetes has become one of the main causes of morbidity and mortality in most countries. It is estimated that 346 million people worldwide have diabetes, and its incidence is arising.

According to the WHO [http://www.who.int/diabetes/en/], diabetes is predicted to become, by 2030, the seventh leading cause of death in the world. Cardiovascular disease represents one of the major complications of diabetes and is responsible for 50% to 80% of early deaths.

A large Danish population-based study conducted on 3.3 million people showed, in diabetic patients requiring glucose-lowering therapy, a cardiovascular risk comparable to nondiabetics who suffered from acute coronary syndrome (ACS), due to which these kinds of patients should receive intensive primary prevention for CVD (antiplatelet therapy, statins, and angiotensin-converting enzyme inhibitor/angiotensin receptor blocker) [1].

The MONICA study also showed a higher incidence of ACS among diabetic patients rather than nondiabetics and that, overall, ACS mortality is four times higher in a male and seven times higher in a female diabetic population [2]. Moreover, a linear positive relationship between admission hyperglycemia and mortality after ACS has been reported. However, in this setting of patients, the optimal management goal of glucose levels still remains uncertain.

The aim of this brief review is to assess the role of a tight glycemic control during ACS in type 2 diabetic subjects, independently from other therapies commonly used for ACS (e.g., statins, antithrombotic therapies, and drug-eluted stents).

2. Rationale for the Goal of Glycemic Control in Diabetic Population

Three studies, ADVANCE [3], ACCORD [4], and VADT [5], have reported unremarkable effects of an intensive glucose lowering on cardiovascular events and overall mortality in type 2 diabetes. Indeed, these trials showed that an intensive therapy performed to gain a too low HbA1c target seems to increase the CV risk. Moreover, an intensive antihyperglycemic therapy increased the risk of severe hypoglycemia.

On the other hand, the UKPDS [6] did not demonstrate a significant reduction of macrovascular events during the intensive treatment, whilst showing that the benefits of an intensive strategy to control blood glucose levels appeared 10 years after the end of treatments [7]. It is outstanding that the UKPDS study population was, with respect to ACCORD, ADVANCE, and VADT studies, younger, with less history of CV disease and neuropathy, lower baseline HbA1c, and lower risk of hypoglycemia.

Actually, in a more recent metaregression analysis [8], a higher BMI, duration of diabetes and incidence of severe hypoglycemia revealed to be associated with a greater risk of cardiovascular death in intensive treatment groups. The same meta-analysis showed that an intensified hypoglycemic treatment in type 2 diabetic patients leads to a significant reduction of the incidence of myocardial infarction, whilst not affecting the incidence of stroke and cardiovascular mortality.

All these findings suggest that the HbA1c target should be set based on the phenotype of diabetic patients like a dress. The International Scientific Society Guidelines have accepted this evidence in order to reduce the CV risk among diabetic people [9].

Unfortunately, less evidences are present for what concerns the impact of a tight glycemic control during acute ischemic events on the short- and long-term CV outcome.

Recent RCTs, which have showed the efficacy of some SGLT2-i and GLP-1 RA (empagliflozin, canagliflozin, and liraglutide) to significantly reduce the CV events in diabetics with history of CVD or at very high CV risk, have a great clinical impact. Moreover, empagliflozin and liraglutide reduced the CV mortality among diabetic people in secondary CV prevention [10–12]. Actually, these findings were not applicable on subjects in primary CV prevention and, above all, in patients with ACS. Within the end of 2018, we expect the results of the DECLARE study, whose aim was to assess the CV effect of dapagliflozin on diabetic patients and also on the primary CV prevention (60% of enrolled population) [13].

Actually, non-RCT showed a protective CV effect by the other classes of antihyperglycemic agents.

In fact, RCTs on DPP4i (saxagliptin, alogliptin, and sitagliptin) showed a noninferiority for the primary endpoint of a composite of death from cardiovascular causes, nonfatal myocardial infarction or nonfatal stroke [14–16]. In particular, alogliptin was originally used in diabetic patients with either acute myocardial infarction (AMI) or unstable angina requiring hospitalization within the previous 15 to 90 days [15]. Moreover, saxagliptin showed an increased rate of heart failure hospitalization [14].

Instead, the addition of empagliflozin and of canagliflozin [10, 11] to the standard of care led to a significant reduction in the hospitalization rates for heart failure compared with placebo (35% and 33%, respectively).

Fascinatingly, the PROactive study showed a not statistically significant 10% reduction in the primary composite endpoint (a combination of cardiovascular disease-driven and procedural events in all vascular beds) versus the statistically significant 16% decrease in the main secondary endpoint (all-cause mortality, myocardial infarction, and stroke) observed with pioglitazone in the secondary CV prevention [17]. Recently, the TOSCA.IT study [18], a long-term, pragmatic trial, showed a similar incidence of cardiovascular events with sulfonylureas and pioglitazone as add-on treatments to metformin.

Because of these and many other evidences, we proposed to critically discuss the literature data regardless of the antihyperglycemic agent used, only selecting the few studies which investigated a strict glycemic control during an ACS.

3. Diabetic Patients after ACS Are at Very High CV Risk

Diabetic patients experience a higher in-hospital mortality and postinfarction complications than nondiabetic ones, such as heart failure, atrial fibrillation, conduction abnormalities, and angina. The poorer outcome among diabetic patients with AMI does not appear to be explained by a larger infarct size. The delayed improvement of both ventricular performance and metabolic disorders at the noninfarcted area level may be responsible for these adverse outcomes, along with an underlying cardiac dysfunction [19].

Many risk factors are involved in the ACS development and progression among which are metabolic syndrome, insulin resistance, hyperglycemia, and oxidative stress [20]. Anyway, a clear understanding of the pathophysiologic mechanisms underlying the infarcted diabetic heart is still missing.

In diabetic patients, metabolic syndrome is associated with a prothrombotic state, involving endothelial dysfunction, hypercoagulability, and a reduced response to fibrinolysis. These complex mechanisms seem to be related to a decreased functional performance of the ischemic organs and a decreased success of both acute and long-term intervention strategies [21]. Among the metabolic risk factors, atherogenic dyslipidemia, associated with an increased number of small dense low-density lipoproteins (LDL), appears to play a predictive role either in the development of cardiovascular events or in the progression of coronary artery disease (CAD) in diabetic patients [22].

4. Role of Insulin Resistance in ACS

Significant evidence supports the theory of a strict relationship between insulin resistance and cardiovascular disease [23]. The insulin effects on inflammatory response, vascular tone, and angiogenesis are attributable to an increased

synthesis of nitric oxide and are deeply reduced in the insulin-resistant states. Insulin infusion, with algorithms aiming to provide an optimal blood glucose control, improves the clinical outcomes of patients with severe acute illness and ACS [24]. Insulin resistance causes a progressive endothelial dysfunction and modifications of glucose and lipid metabolism, establishing a continuous negative feedback cycle and eventually leading to an acute vascular damage [23]. Actually, both insulin resistance and hyperglycemia seem to play important roles in the pathogenesis of ACS.

5. Effects of Hyperglycemia during ACS

Several studies identified hyperglycemia as an independent risk factor for diabetic cardiomyopathy, through cardiac cell apoptosis [25]. Apoptotic myocyte loss could represent an important mechanism leading to a poor prognosis after AMI in diabetic patients as it contributes to a progressive cardiac remodeling, through left ventricular enlargement and interstitial fibrosis, resulting in an increased synthesis of type III collagen by cardiac fibroblasts [26].

Abnormal glucose tolerance is almost twice among patients with an ACS, as in population-based controls [27]. Hyperglycemia acts as a multiplier of cardiovascular risk and is implicated in vascular damage and cardiac myocyte death through different molecular mechanisms: advanced glycation end products (AGE), protein kinase C (PKC), polyol pathway flux, and the hexosamine pathway. All of these reflect a single hyperglycemia-induced process of overproduction of superoxide by the mitochondrial electron transport chain [20].

More fascinatingly, in a high-risk intensive cardiac care unit general population (only 17% had known diabetes), both hyperglycemia at admission (glucose ≥ 9 mmol/L) and sustained hyperglycemia during hospitalization (average glucose levels ≥ 8 mmol/L) were independent predictors of all-cause mortality [28]. Similar relationships between admission glucose levels and hospital mortality were also reported in other studies [29].

ACS results in many systemic metabolic changes, particularly evident in diabetic patients with an already reduced capability of insulin secretion and use of glucose for the production of energy. Clinical and experimental evidences suggest that the sympathoadrenal activation contributes to mortality in patients with ischemic heart disease and the magnitude of the adrenocortical response is governed by the amount of myocardial necrosis [30]. An excessive catecholamine activity, through a glycogenolytic effect, contributes to a rise in blood glucose levels. In addition, adrenaline is a powerful suppressor of the normal insulin response to a glucose load.

The main result of these hormonal pathways is an increased turnover of FFAs. In well-oxygenated hearts, FFAs have been identified as the preferred substrate by both *in vivo* and *in vitro* studies, accounting for 35% to 75% of oxygen consumption. In hypoxic hearts, FFA oxidation is suppressed and glycolysis stimulated, leading to an increase of triglyceride levels. Experimental and clinical observations suggest that increased circulating concentrations of FFAs may be associated with an adverse outcome of ACS [31], by means of several mechanisms such as direct toxicity, increased oxygen demand, and direct inhibition of glucose oxidation. These metabolic changes may play a role in the development of arrhythmias and disorders of conduction. This relationship could be explained by two mechanisms: stimulation of the hypoxic myocardium by increased circulating catecholamine and increased myocardial oxygen requirement resulting from the utilization of FFAs as an energy substrate [32].

Recently, several mechanisms showed how high FFA concentrations may be toxic in acute ischemic myocardium, such as mitochondrial uncoupling, activation of lipids in the mitochondria, inhibition of β-oxidation, inhibition of the Na^+-K^+-ATPase pump leading to high intracellular sodium and calcium, or GLU-4 reduction causing reduced insulin-stimulated glucose transport [32]. Thus, monitoring and reducing concentrations of FFAs during and after an ACS represent a priority [33].

Recently, it was confirmed that the FFA level might be a predictor of the severity of myocardial ischemia during the subacute onset of ACS attack and was observed that the FFA levels increased with the severity of necrosis and ischemia, such as cTnT [31]. In the same paper, an association between WBC counts, hs-CRP, and FFA levels was observed in ACS, suggesting a possible mechanism relating FFAs together with inflammatory factors affecting the progress of ischemia. Moreover, elevated circulating FFA levels led to endothelial dysfunction *in vivo* through the activation of PKC-mediated inflammatory pathways and an excessive generation of oxidants [34, 35], which would partially explain a proarrhythmogenic activity of FFAs.

6. Are There Clinical Evidences for Tight Glycemic Control during ACS?

A reduction in free fatty acid plasma levels and an increased availability of glucose can be achieved by using a glucose-insulin-potassium infusion (GIKi) during AMI. The GIKi is associated with an improvement of either long-term prognosis or left ventricular mechanical performance [36]. Moreover, early after AMI, high-dose GIK infusion improves the cardiac function, as confirmed by hemodynamic measurements. In fact, high-dose GIK can decrease the cardiomyocyte apoptosis in AMI patients with reperfusion therapy [37]. Moreover, high-dose GIK could improve cardiac remodeling in AMI patients receiving primary PCI by lowering vascular resistance [38].

As a confirmation of this hypothesis, the DIGAMI study (Diabetes Mellitus, Insulin Glucose Infusion in Acute Myocardial Infarction) showed that GIKi administration in the early 24 hours after acute myocardial infarction (AMI), followed by a multidose subcutaneous insulin regimen, facilitates a persistent improvement of glucose control and reduces the long-term mortality in diabetic patients. In particular, the relative mortality reduction reduced by 29% after 1 year. Interestingly, this particularly manifested in patients with a low cardiovascular risk profile and no previous insulin

treatment [39]. Actually, the DIGAMI study has brought too many criticisms, such as the uncertainty whether the GIK infusion during AMI or the followed long-term insulin treatment caused the favorable long-term outcome, the small simple size, large confident intervals and the potential bias resulted with only the 50% of all eligible patients being randomized.

The DIGAMI 2 trial was planned and conducted to further investigate the possible effects on mortality and morbidity of an insulin-based management on diabetic patients with AMI. In this trial, three treatment strategies were compared: acute insulin-glucose infusion followed by insulin-based long-term glucose control; insulin-glucose infusion followed by standard glucose control and routine metabolic management according to local practice. The study did not confirm the usefulness on the overall survival rate due to an early and long-term insulin treatment in type 2 diabetic patients following AMI. In fact, neither an acutely introduced long-term insulin treatment did not improve survival in type 2 diabetic patients following myocardial infarction when compared with a conventional management at similar levels of glucose control nor an insulin-based treatment lowers the number of nonfatal myocardial reinfarctions and strokes [40]. In particular, DIGAMI 2 did not show any mortality benefit in a maximum follow-up time of up to 3 years. However, these results suggested blood glucose levels as a significant and independent mortality predictor among diabetic patients, enhancing the important role of glucose control in their management.

Moreover, a post hoc analysis of DIGAMI 2 [41], adjusting for confounders such as glycemic control, did not show any significant difference in mortality among sulphonylureas, metformin, and insulin. However, the risk of nonfatal myocardial infarction and stroke was significantly increased by insulin treatment, whilst metformin was protective.

The most reasonable reason for the difference between DIGAMI 1 and 2 findings is that in DIGAMI 2, changes in glucose concentrations between control and insulin treatment groups were nonsignificant, despite the intent to obtain target-driven, strict glycemic control in patients assigned to the insulin-based groups in these trials. Moreover, HbA1c at admission was substantially higher in DIGAMI 1 than in DIGAMI 2 (HbA1c 8.2% vs 7.2%). Interestingly, findings from the recent 20-year follow-up of the DIGAMI 1 cohort supported that insulin-based intensified glycemic control after acute myocardial infarction increased survival, with a lasting effect of at least 8 years [42]. In particular, contrarily to the favorable effects observed in patients with no previous insulin use and at a low cardiovascular risk, in whom longevity was prolonged most, from 6.9 years to 9.4 years, intensified insulin-based glycemic control did not affect the outcome in patients at high risk and no previous insulin treatment. These findings seem to support the conclusions from the ACCORD and ADVANCE trials, which demonstrated that a tight glycemic control in patients with long-standing diabetes and advanced cardiovascular disease does not improve mortality.

7. Mechanisms Supporting the Protective Role of Tight Glycemic Control during ACS

Accumulating evidence supports the hypothesis that the heart has a pool of cardiac stem-progenitor cells (CSCs), which can differentiate into cardiomyocytes and acutely populate the damaged regions of ischemic myocardium, regenerating coronary vessels [43]. In particular, Anversa and coworkers proposed a classification of cardiac immature cells into 4 classes: cardiac stem cells (CSCs), progenitors (CPCs), precursors (MPCs), and amplifying cells. These cell types may be considered as subsequent steps in the progressive evolution from a more primitive to a more differentiated phenotype [44].

There is evidence that diabetes plays an important role in the dramatic loss of MPC function in animal models. The high levels of oxygen reactive species, produced by hyperglycemia during AMI, result in the inhibition of both cell replication and differentiation, thus favoring the development of a cardiac myopathy characterized by a decrease in muscle mass and impaired ventricular function [45]. Both MPC number and myocyte proliferation significantly increase when a tight glycemic control is achieved in the early stage of AMI. A tight glycemic control during an acute ischemic damage is associated with an increased regenerative potential of the myocardium [31]. Glucose control may have more important results than insulin treatment in the improvement of the cardiac outcome among diabetic patients.

In 2011, Samaropoulos and coworkers demonstrated how an intensive glycemic control in middle- to old-aged type 2 diabetic patients, who already had or are at risk for cardiovascular disease, was associated with a reduction in high-sensitivity C-reactive protein (hs-CRP) [46].

This finding suggests that an increased inflammatory immune process seems to be most likely a mechanism linking acute hyperglycemias to poor cardiac outcomes in AMI patients [47]. Inflammatory response and cytokine elaboration are particularly active after AMI and contribute to cardiac remodeling, through progressive myocyte apoptosis, hypertrophy, and defects in contractility [48].

Recently, Tatsch et al. showed an association between a poor control of type 2 diabetes and increased levels of oxidative, inflammatory, and endothelial biomarkers, resulting in DNA damage [49].

High glucose levels have been reported to enhance inducible nitric oxide synthase (iNOS) expression, leading to the production of high levels of nitric oxide (NO) [50]. Moreover, iNOS is expressed in the myocardium after MI. Although NO may have beneficial effects on the inflammatory response and the vascular resistance, increased NO levels contribute to the production of peroxynitrite, hence producing a myocardial damage and a higher mortality after AMI [51].

In addition to hyperglycemia, oxidative stress may be induced by soluble advanced glycation end products (AGE). Among AGE precursors, methylglyoxal (MG) is considered as one of the key intermediates linking hyperglycemia and intensive lipolysis, two dominant metabolic changes in diabetes [52]. Oxidized low-density lipoprotein (oxLDL) in

diabetic patients enhances monocyte chemoattractant protein-1 (MCP1) gene expression in endothelial cells, increasing the atherogenic process and promoting endothelial dysfunction [53].

Moreover, deleterious vascular effects of endothelial dysfunction are associated with smooth muscle cell proliferation after vascular injury, including injury from catheter-based interventions. In fact, diabetic patients have a greater incidence of restenosis after percutaneous coronary intervention (PCI), related to an exaggerated tissue proliferation in lesions treated either with or without stents. The restenosis process begins very early, between 1 and 3 months after coronary angioplasty [54]. Timmer and colleagues examined the effects of a periprocedural tight glycemic control during PCI on the restenosis rate in hyperglycemic patients with ST segment elevation myocardial infarction (STEMI), showing that both elevated glucose admission and HbA1c levels are associated with adverse outcomes [55].

8. Evidences for Tight Glycemic Control in Surgical and Critically Ill Patients

Several studies, though not conducted in patients with acute coronary syndrome, partially confirmed this relationship between CV outcome and glycemic *milieu*. A tight glycemic control during the perisurgical period seems to decrease the inflammatory immune reaction, as well as both nitrotyrosine levels and MCP1, hence driving to a better prognosis [56]. In reality, insulin resistance during surgery, rather than the presence of diabetes mellitus, is associated with an increased risk of major complications.

It is well known that major surgical tissue trauma leads to alterations in glucose metabolism, resulting in hyperglycemia and insulin resistance. This could be explained by specific neuroendocrine changes, such as increased circulating concentrations of cortisol, glucagon, and catecholamine. The extent of insulin resistance during surgery depends on the intensity of trauma, suggesting insulin resistance as a marker of surgical stress, with potential relevance for the clinical outcome [57]. The number of patients who suffered a major complication and an increased rate of superficial wound infections significantly increased in diabetics with poor preoperative glycemic control when compared with nondiabetics.

Van den Berghe and colleagues obtained similar results studying patients treated in an intensive care unit (ICU) [58]. They asserted that an intensive insulin therapy during intensive care prevents morbidity, though not significantly reducing mortality. More recently, Brunkhorst et al. [59] confirmed that there are no significant differences in the death rate for the insulin intensive-treated group and showed that the use of intensive therapy in critically ill patients determines an increased risk for serious adverse events related to hypoglycemia.

Finally, the NICE-SUGAR study, a large, international, randomized trial [60], suggested that a goal of normoglycemia for glucose control does not necessarily benefit critically ill patients, rather being harmful, due to a major incidence of deaths from cardiovascular causes in the intensive control group compared to that resulted in the conventional control group. Moreover, the former group shows a lower median survival time than the latter. In particular, it was observed that an intensive glucose control increased mortality among adults in ICU: a blood glucose target of 180 mg/dL or less resulted in lower mortality than a target of 81 to 108 mg/dL.

9. Position Statements of Scientific Societies

All these studies confirm that hyperglycemia is common during ACS and is associated with increased mortality rates. Anyway, it remains still unclear, as stated by the American Heart Association, whether hyperglycemia is either a marker or a mediator of higher mortality and whether hyperglycemia treatment improves outcomes [61].

Correctly, Scientific Societies, underlying the absence of robust data for an optimal glucose management (e.g., treatment thresholds and glucose targets) in STEMI patients, suggested a close, though not too strict glucose control as that of the best approach.

The last ESC task force on diabetes and CV diseases developed in collaboration with EASD suggested, according to DIGAMI 1, that DM and AMI would benefit from glycemic control, in the case of a significant hyperglycemia (higher than 10 mmol/L or 180 mg/dL), with the target adapted to possible comorbidities as a class 2a recommendation. In particular, an approximation towards normoglycemia with less stringent targets, in those with severe comorbidities, would represent a reasonable goal, though exact targets have still to be defined. Moreover, the two Scientific Societies stated insulin infusion as the most efficient way to rapidly achieve glucose control [62].

More recently, the 2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST segment elevation [63] stated, as class 2a recommendation, that glucose-lowering therapy should be considered in ACS patients with glucose levels > 10 mmol/L (>180 mg/dL), whilst episodes of hypoglycemia (defined as glucose levels ≤ 3.9 mmol/L or ≤ 70 mg/dL) should be avoided.

Finally, the 2018 ADA guidelines [64] recommended, in hospitalized patients, a glucose target range between 140 mg/dL and 180 mg/dL (7.8–10.0 mmol/L) for the majority of critically ill patients (level A of evidence). Clinical judgment, combined with the ongoing assessment of the patient's clinical status, should be incorporated into the day-to-day decisions regarding insulin doses. Remarkably, the treatment regimen should be reviewed and changed as necessary to prevent further hypoglycemia when a blood glucose value is ≤ 70 mg/dL (3.9 mmol/L) (level C of evidence).

Notably, whilst all these guidelines do not seem mandatory with regard to a strict hyperglycemic cutoff, instead, they agree to absolutely avoid hypoglycemia. Therefore, according to the recent joint position statement of the American Diabetes Association and the European Association for the Study of Diabetes [65], the hypoglycemia alert value in hospitalized patients has to be defined as blood glucose ≤ 70 mg/dL (3.9 mmol/L) and clinically significant hypoglycemia as glucose values < 54 mg/dL (3.0 mmol/L),

to be reported in clinical trials. This statement thus represents a good threshold for future studies.

10. Conclusions

In conclusion, optimal glucose target levels and treatment regimens in this setting of patients are still under debate. Future studies on intensive glucose control must be developed to reduce both glucose variability and risk of hypoglycemia, thus achieving an optimal blood glucose concentration in critically ill patients, particularly in ACS.

Conflicts of Interest

The authors exclude any conflict of interest.

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Hindawi
Journal of Diabetes Research
Volume 2018, Article ID 2742565, 8 pages
https://doi.org/10.1155/2018/2742565

Review Article

Diabetes and Associated Cardiovascular Complications in American Indians/Alaskan Natives: A Review of Risks and Prevention Strategies

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Received 25 April 2018; Accepted 12 August 2018; Published 13 September 2018

Academic Editor: Celestino Sardu

Diabetes mellitus (DM) is the seventh leading cause of death in the United States and the leading cause of death in the U.S. American Indian/Alaskan Natives (AI/ANs), who comprise only 2% of the total population. The AI/AN population has a high prevalence of DM in adults aged 20 years or older and is developing DM at a younger age than the general U.S. population. DM is a major risk factor for cardiovascular disease (CVD), and mortality from CVD is higher in AI/ANs than the general population, as is the prevalence of stroke and 1-year poststroke mortality for both genders when compared to non-Hispanic whites. A genome-wide scan found a number of chromosome linkages in the AI/AN population that suggest that genetic factors may contribute to their high risk of DM and CVD. Importantly, studies also suggest that in addition to race/ethnicity, cultural norms and historic conditions play important roles in the prevalence of DM and CVD in this population. Therefore, multiple factors should be taken into consideration when establishing prevention programs to decrease the prevalence of obesity, diabetes, and CVD incidence among adults and children in the AI/AN population. Prevention programs should focus on behavioral risk factors and lifestyle changes like encouraging smoking cessation, healthy diet, and increased physical activity while taking into consideration cultural, economic, and geographic factors.

1. Introduction

Diabetes mellitus (DM) is the seventh leading cause of death in the United States. According to the 2017 National Diabetes Statistic report, 9.4% (30.3 million people) of the U.S. population are affected by diabetes and 33% of U.S. adults aged 18 years and older have prediabetes [1]. DM is a major risk factor for cardiovascular disease (CVD), with DM patients being two to four times more likely to develop CVD than the general population [2]. In addition, both DM and insulin resistance are known to increase the risk of developing heart failure independent of age, gender, coronary artery disease (CAD), or hypertension and are associated with worse clinical outcomes [3]. Furthermore, CVD is the leading cause of morbidity and mortality in those diagnosed with DM, and the mortality rate from heart failure is almost doubled in patients who have diabetes when compared to their normal glycemic counterparts [4].

Although diabetes affects all races, the Centers for Disease Control and Prevention (CDC) report on DM indicates that the American Indian/Alaskan Native (AI/AN) populations, who comprise only 2% of the U.S. population, have a higher prevalence of DM in those aged 20 years or older, with a prevalence more than two times that of the general population [5]. DM is the fourth leading cause of death in the AI/AN population [3]. Additionally, AI/AN youths are disproportionately affected by obesity, a major risk factor for DM, compared to other ethnic groups in the United States, with 40 to 50% of children in many AI/AN communities being reported as overweight or obese [6, 7]. As is the case in the U.S. population in general, CVD is also the leading cause of death in the AI/AN population [8, 9].

However, the incidence of CVD in this population shows considerable variability, ranging from 15 to 28 per 1000 among AI/AN men and only 9 to 15 per 1000 in AI/AN women ages 45–74 [10–12], and the factors for this discrepancy remain unclear.

To date, there have been very few comprehensive reviews of the literature on the prevalence of DM and DM-related CVD complications among the AI/AN population. In the current review, we summarize findings on the prevalence of DM and CVD, analyze the risk factors contributing to the high prevalence of these diseases in the AI/AN population, discuss strategies for disease prevention, and address the existing health disparities.

2. The Prevalence of DM Is High in the AI/AN Population and Is Associated with Younger Age of Onset and Higher Risk of Developing CVD

2.1. The Prevalence of DM Is High in AI/AN Populations and Increasing, with a Trend toward Younger Age of Onset. The prevalence of DM is consistently high in AI/AN populations of all ages and genders [13–17] and is almost three times that of U.S. non-Hispanic whites when age is adjusted [10]. In addition, an alarming increase in DM prevalence among AI/AN was revealed in the Behavioral Risk Factor Surveillance System (BRFSS surveys) which found an increase of 29% in the incidence of DM between 1990 and 1997 in the AI/AN population [18]. A similar increase in DM prevalence among AI/ANs was also observed by the BRFSS from 2000 to 2006, as well as a similarly increasing trend compared to non-Hispanic whites from 2004 to 2008 observed in data from the National Health Interview Survey (NHIS) [10]. However, the increased rate of DM prevalence in the AI/AN population has large discrepancies from region to region, ranging from 16% in the Northern Plains to as high as 76% in the Alaskan region based on the Indian Health Services (IHS) national outpatient database [10]. Specifically, the prevalence of DM increased by 47% in AI/AN adults 20–24 years old and by 50% for those 25–34 years of age [19]. The prevalence of DM in older adults (>55 years) was also higher compared to that of non-Hispanic whites (21.9% and 13.0%, respectively) based on BRFSS aggregated data from 2001 to 2004 [20]. Importantly, an association with younger age of individuals who develop DM among AI/AN populations compared to the U.S. diabetic population has been identified [10], with the number of children, adolescents, and young adults diagnosed with diabetes increasing by 71% based on the IHS national outpatient database from 1990 to 1998 [19]. AI/AN women had the highest prevalence of DM among the U.S. population [21, 22], with 5.4% of AI/AN women between 18 and 44 years of age affected (BRFSS data 2005–2007) [10], compared to only 2.2% of non-Hispanic whites [23]. Data from the Well-Integrated Screening and Evaluation for Women Across the Nation (WISEWOMAN) study confirmed this finding [24]. It should be mentioned that although the advantage of national survey data is admitted, these self-reported data probably have significant biases which should be taken into consideration when making comparisons between populations.

Regional variations in DM control and morbidity among AI/AN populations have also been noted. For instance, 27% of DM patients under age 45 have plasma hemoglobin A1c levels greater than 9.0 in the Alaskan region, versus 56% in the Southwest AI population [25]. Variations in DM prevalence and diabetes control among older individuals were also obvious in these regions [25]. AI adults with DM were also more likely to present with diagnoses of hypertension, renal failure, lower extremity amputations, neuropathy, mental health disorders, and substance abuse with comorbid liver disease in the Phoenix area compared to other U.S. adults with DM [26].

2.2. AI/AN Populations Have a High Risk of Developing CVD and an Associated Increase in CVD Mortality. Heart disease is the leading cause of death among AI/AN populations, with CVD resulting in 1813 deaths in 2009 [27] and 3288 in 2014 [28]. Self-reported heart disease, defined as the existence of coronary heart disease (CHD), angina, heart attack, or other heart conditions or diseases in the AI/AN population, is higher than in Non-Hispanic whites (14.7% compared to 12.2%, respectively) [29]. These disparities in the prevalence of CVD among AI/AN populations were also seen in a number of other studies [30–34].

The Strong Heart Study (SHS), supported by the National Heart, Lung, and Blood Institute (NHLBI), is the largest epidemiological study yet conducted to have examined cardiovascular risk factors in the AI population [35]. The SHS revealed that cardiovascular mortality was higher in AI populations compared to the general U.S. population [13, 36]. Similarly, registration data with the National Death Index indicated that the mortality rate from heart disease was significantly higher among AI/ANs than whites aged 35 years and older from 1990 to 2009 [31, 37]. The incidence of congestive heart failure (CHF) among AI/AN men, however, was higher than AI/AN women and was associated with a worse prognosis. In agreement with these findings, further analysis of echocardiograms from participants in the SHS aged 45 to 74 years old revealed that AI/AN women have much better left ventricular (LV) contractility and greater LV myocardial and chamber function than AI/AN men [38–42].

2.3. Type 2 Diabetes Is Associated with a High Prevalence of CVD Complications in the AI/AN Population. It has been well documented that type 2 diabetes (T2D) patients have a greater risk for cardiovascular morbidity and mortality in the general population [43]. Recent research suggests that cardiac death, rate of adverse cardiovascular outcomes such as readmission for acute coronary syndrome (ACS), and heart failure were higher in patients with DM [44]. DM patients with nonobstructive coronary artery stenosis (NOCS; 20%–49% luminal stenosis) after a first non-ST-elevation myocardial infarction (NSTEMI) had a worse prognosis. However, treatment with a GLP-1 analogue was proven to result in a significant improvement on clinical outcomes [44]. The association between autonomic dysfunction and silent atrial fibrillation (AF) was also observed in T2D

patients younger than 60 years in one study [45]. Furthermore, several studies suggest that DM has direct adverse effects on cardiac structure and function independent of the severity of CAD but is highly associated with increased LV wall mass, LV torsion, and decreased myocardial perfusion [46]. This correlation was also observed in the AI/AN population [42, 47–51]. A multinational study comparing the vascular disease incidence in younger diabetic patients who were diagnosed with DM before the age of 30 revealed that AI men had higher rates of renal failure and lower extremity amputation than other ethnic groups [52]. The same study also reported higher incidences of retinopathy, clinical proteinuria, and albuminuria in that population.

It has been shown that systemic hypertension (SH) increases the risk of CVD in diabetic patients [53]. DM and SH were both significantly associated with adverse effects on LV wall structure and function in the AI population after adjusting for age, gender, BMI, and heart rate [54]. Furthermore, the impact of DM and SH, when combined, was associated with more severely abnormal LV relaxation, a greater degree of LV hypertrophy, myocardial dysfunction, and arterial stiffness than either condition alone in AI/AN populations [54].

2.4. AI/AN Populations Have a Higher Risk of Stroke and Poststroke Mortality. CHD and stroke share several risk factors such as hypertension, smoking, diabetes, physical inactivity, and obesity. Patients with CAD have an increased risk of developing stroke. Recent studies suggest that subclinical episodes of atrial fibrillation (AF) occurred frequently in T2D patients and are associated with an increased risk of silent cerebral infarct (SCI) and the development of stroke in T2D patients who are younger than 60 years of age [55]. Additionally, 9% of the diabetic patients developed stroke even if they were treated with antiplatelet medications [55]. Stroke is the seventh leading cause of death among AI/ANs, and the mortality rate from stroke is also higher than that of whites (29.5 per 100,000 for AI/ANs compared to 24.0 per 100,000 for whites) [56, 57]. Furthermore, the one-year poststroke mortality rate in AI women (33.1%) and AI men (31%) is higher than in the general population for both genders (24% for women and 21% for men, respectively) [58, 59].

When compared to the non-Hispanic white population, the prevalence of stroke in AI/ANs was higher (2.4% and 4.7%, respectively), with a concomitantly higher risk ratios than whites for all three stroke subtypes [36]. Those SHS participants who had not had a stroke at baseline (from 1989 to 1992) were followed until the end of 2004, and the incidence of stroke was found to be much higher in AI/AN participants [36].

3. Genetics, Heritability, and Other Risk Factors Contribute to the Increased Incidence of CVD in the AI/AN Population

3.1. Genetic Factors and Heritability Contribute to the High Risk of DM and CVD in the AI/AN Population. Genetic factors and heritability may play an important role in the high prevalence of CVD observed in the AI/AN population. To explore this possibility, SHS initiated a genetic epidemiology study and a full family study to explore the potential impact of genetic factors and heritability on the prevalence of these disorders in the AI/AN population [60, 61]. The SHS pilot family study recruited 10 large families from each of three field centers in addition to the original cohort. Subsequently, approximately 30 families (10 per field center) with more than 900 family members participated in the study. The CVD risk factors and heritability were also investigated. Localization of genes that contribute to CVD risk was conducted though linkage analysis. This family study, conducted from 2001 to 2003, recruited an additional 18 to 25 extended families (a total of about 900 members at least 15 years of age) from each of the field centers, with a total of 3776 subjects from 94 families, of whom 825 were SHS pilot family participants (https://strongheartstudy.org/Research/ResearchDesign.aspx). A genome-wide scan found chromosome linkages for a number of pathological conditions that are more prevalent in the AI/AN population. For example, the scan identified significant linkage of a locus on chromosome 4q35 to weight and BMI for 963 individuals from 58 families from Arizona, Oklahoma, and North and South Dakota [62]. Further analyses of individual study sites revealed the greatest linkage between loci on chromosome 4 to obesity in an AI population from Arizona. Linkage signals for BMI were also observed on chromosomes 5, 7, 8, and 10 in another study [62]. Genome-wide linkage analysis also identified a significant linkage of a locus for the left carotid artery diastolic and systolic lumen diameter. For instance, a linkage of a locus on chromosome 7 at 120 cM for the left carotid artery diastolic and systolic lumen diameter was identified in the Arizona SHFS participants and a linkage of a locus on chromosome 12 at 153 cM for the left carotid artery diastolic and systolic lumen diameter was found in the Oklahoma SHFS participants [63]. Additionally, a linkage for the right carotid artery diastolic and systolic lumen diameter was also detected on chromosome 9 at 154 cM in the Oklahoma participants [63]. Linkages of chromosome 7p19q with pulse pressure, glucose/insulin/obesity factor to chromosome 4, the dyslipidemia factor to chromosome 12, and the blood pressure factor to chromosome 1 were also identified among SHFS participants [64, 65].

In several studies, North et al. have found evidence for the effect of heritability on the prevalence of DM and CVD in the AI/AN population. For instance, significant heritability of diabetes status [66], as well as several CVD risk factor phenotypes like high density lipoprotein cholesterol and diastolic blood pressure, was demonstrated among AIs [61]. In addition, significant heritability for the common carotid artery diastolic diameter, intimal-medial wall thickness, vascular mass, arterial stiffness, and the augmentation index has been identified in the AI population [60].

3.2. Risk Factors Contributing to the Increased Incidence of CVD in the AI/AN Population. Evidence suggests that several risk factors, such as tobacco abuse, DM, hypertension, and elevated cholesterol levels, contribute to the increased incidence of CVD in the AI/AN population [11, 35]. Analysis

of the chronic heart disease (CHD) outcome data from SHS revealed that age, gender, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, smoking, DM, hypertension, and albuminuria were significant CHD risk factors in this population [35, 67]. Interviews conducted through the BRFSS of AI adults living in the seven Montana reservations showed an alarmingly high level of modifiable CVD risk factors among those with and without DM [68, 69]. Specifically, tobacco use is a big concern in this population with 26.7% of AI/AN adults identifying as smokers compared to only 15.2% of Asian men, 17.3% of Hispanic men, 23.7% of non-Hispanic black men, and 23.9% non-Hispanic white men, after age-adjusted estimates [70]. Similarly, AI/AN women (20.7%) are more likely to be current cigarette smokers than Asian women (5.5%), Hispanic women (9.6%), non-Hispanic black women (17.6%), and non-Hispanic white women (20.9%) [12]. Physical inactivity is another important risk factor that contributes significantly to CHD development. In 2011, only 17.0% of AI/AN adults (age 18 and older) met the 2008 federal guidelines for physical activity [71]. Obesity or being overweight is another risk factor for CVD prevalent in the AI/AN and general population. Only 27.6% of AI/AN individual maintain a healthy weight; a rate much less than whites (36.6%) or Asians (56.7%) [36].

4. Prevention of DM and CVD in the AI/AN Population

4.1. Control and Management of Risk Factors for DM and CVD. Physical inactivity, obesity, and hypertension are known major risk factors for DM and CVD. Among these, physical inactivity is modifiable and obesity is a controllable risk factor. DM and CVD are preventable by controlling physical activity and obesity. It is well recognized that exercise and weight loss reduce blood pressure, increase insulin sensitivity, prevent or delay the onset of T2D, lower CVD risk, and reduce the risk for heart attack and stroke [72]. Successful management and control of these modifiable risk factors have been the focus for public health, and it has been shown to reduce the risk of DM and CVD as well as delay its progression and complications, including CVD, in the general population [73]. Furthermore, studies have shown the positive impact of lifestyle interventions on preventing DM and CVD risks in the AI/AN population [74]. For instance, early lifestyle interventions that focused on promoting healthy eating and regular exercise were shown to decrease the development of metabolic syndrome in the Southwestern AI population [74]. A targeted tri-weekly exercise regimen, coupled with nutritional counselling, conducted in a community-based cohort study among 65 Zuni Pueblo adolescents resulted in significant improvements in BMI, fat-free mass, total body fat, and fasting lipid profile after six months [74].

4.2. Recognition and Integration of Specific Health Issues of Individual AI/AN Communities into Health Promotion Programs. Substantial variations in the prevalence of DM and CVD among the AI/AN population when compared to the general population have been identified. This fact suggests that race, ethnicity, cultural norms, and historic conditions play an important role in the prevalence of DM and CVD. Recognizing these disparities and incorporating the health issues specific to each community into health promotion programs and policies are a critical step in overcoming the disproportionate toll these illnesses take on a population [75]. Food insecurity, defined as uncertain or inadequate access to enough foods for an active healthy life due to shortage of money or resources, has been linked to obesity in children, DM in adults, and poor glucose control in DM patients [76]. Unfortunately, 23% of AI/AN families had incomes below the poverty level in 2010, compared to 16% of the general population. One study has shown that a high level of social integration was significantly correlated with diabetes management behaviors, including monitoring glucose levels and A1C, maintaining a healthy diet, participating in a regular exercise program, and examining feet [73]. In addition, most AI/AN communities live in rural areas sometimes in isolated (e.g., Alaskan villages) living circumstances, which is a huge barrier to healthy eating due to high cost and long commutes for fresh food [77]. Food insecurity was reported in 40% of households in a study conducted in the rural Northern Plains reservation [78]. Therefore, providing both affordable nutrient-rich food choices near AI/AN communities and dietary intervention at the individual level should be included in environmental intervention plans.

4.3. The Special Diabetes Program for Indians (SDPI) with DM Prevention. In order to address the diabetes epidemic among the AI/AN population, congress initiated the Special Diabetes Program for Indians (SDPI) in 1997 to provide funds for DM prevention and treatment and to increase access to quality diabetes care with a focus on effective evidence-based intervention strategies. The SDPI diabetes best practices' program focuses on screening and monitoring glycemic control, blood pressure, cardiovascular complications, retinopathy, gum and tooth diseases, depression, tobacco use, nutrition, and physical activity education [79]. The SDPI diabetes prevention and healthy heart program focuses on translating scientific evidence into prevention and reduction of diabetes and CVD risk factors among AI/AN communities. The SDPI diabetes prevention grant program is designed to reduce DM risk in high-risk individuals through a proven lifestyle change intervention, and the SDPI healthy heart (SDPI-HH) program seeks to reduce CVD risk among AI/ANs with T2D [80]. This program provides standardized health care services for cardiovascular risk reduction which includes assessment of blood sugar and blood pressure, as well as encouraging patient exercise and healthy nutrition [81]. The SDPI-HH model engages patients in the health care team and provides a promising framework for understanding barriers to and solutions for improving health care. Finally, it strives to overcome medical mistrust and addresses the specific health problems faced by the AI/AN population [81].

The SDPI has had a positive impact on prevention and control of diabetes among AI/AN populations (presented in the SDPI 2014 Report to Congress). The increase in diabetes

prevalence among AI/AN adults has slowed and has not increased in AI youth, suggesting that key diabetic clinical parameters are under control and more importantly, the incidence of end-stage renal disease in people with diabetes is decreasing [82–86].

4.4. Improving CVD Knowledge and Health Literacy Levels in the AI/AN Population. Medical mistrust is defined as a patient feeling uncomfortable, fearful, or suspicious in a health care setting and has been found to be significantly higher in AI/AN populations when compared to whites [87]. Health literacy (HL), which is the ability to acquire, process, and comprehend basic health information, may also have significant implications in medical mistrust. Individuals with inadequate HL skills have been shown to have more restricted knowledge of a variety of health conditions and medical services including DM, chronic heart failure (CHF), and hypertension [88]. 48% of AI/AN adults have limited HL skills compared to 36% of U.S. adults. This discrepancy is likely to be the result of limited educational attainment and a high poverty rate in AI/AN populations [67, 89, 90]. One specific area of concern is knowledge about CVD, which has been shown to be restricted in AI/ANs. For instance, AI/AN knowledge of heart attack and stroke symptoms was more limited than that of the general population [91]. Although they understand the risk of obesity and importance of physical inactivity in CVD development, they are unable to tell what blood pressure values would be considered high [91, 92]. The National Heart, Lung, and Blood Institute (NHLBI) developed the Honoring the Gift of Heart Health (HGHH) curriculum to focus on improving the HL level of AI/AN communities. Improvements in HL levels, including heart attack knowledge, stroke, and general CVD knowledge, have been observed in HGHH curriculum participants [92].

In conclusion, the incidence and prevalence of DM and CVD are much higher in the AI/AN population when compared to any other racial or ethnic groups in the U.S. The high risk of developing DM and CVD complications is also disproportionately higher irrespective of age, gender, or geographical location. Multiple risk factors should be considered when establishing prevention programs to decrease the prevalence of obesity, diabetes, and CVD among adults and children in the AI/AN population. Prevention programs should focus on behavioral risk factors and lifestyle changes such as encouraging smoking cessation, eating a healthy diet, and increasing physical activity. In addition, cultural norms, historical conditions, and individual health issues among AI/AN communities should be acknowledged and integrated into all prevention programs.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Anil Poudel and Joseph Yi Zhou contributed equally to this work.

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Hindawi
Journal of Diabetes Research
Volume 2018, Article ID 1780683, 6 pages
https://doi.org/10.1155/2018/1780683

Research Article

The Impact of Type 2 Diabetes Mellitus on Long-Term Prognosis in Patients of Different Ages with Myocardial Infarction

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Received 15 February 2018; Revised 22 May 2018; Accepted 24 June 2018; Published 10 July 2018

Academic Editor: Gaetano Santulli

The objective of the study was to assess the impact of DM2 at baseline on long-term mortality after acute myocardial infarction (MI) among different age groups. The data were taken from: "Register of Acute Myocardial Infarction." A total of 862 patients were followed for five years after acute myocardial infarction. The primary endpoint was death from any cause. The patients were categorized into 2 groups based on their ages: group 1—comprised patients older than working age ($n = 358$) and group 2—comprised employable patients ($n = 504$). A total of 208 patients were diagnosed with both cardiovascular disease and DM2. Elderly patients with DM2 had worse prognosis and increased five-year mortality compared with patients of the same age group without DM2. Statistically significant differences in long-term outcomes were found in adult patients ($p = 0.004$) only in group with longer duration of diabetes, unlike the group with DM2 onset. In conclusion, Type 2 DM increased 5-year mortality rate of elderly patients with myocardial infarction. However, younger patients with both myocardial infarction and DM2 had more complications in the early post-MI period compared with patients of the same age group without DM2 but did not show any statistically significant differences in the long-term outcome.

1. Introduction

Modern achievements in medical science and practice allowed us to obtain tremendous success in the treatment of chronic noncommunicable diseases. Improved prognosis and quality of life in patients suffering from the most common socially significant pathology of the circulatory system—ischemic heart disease—contribute to the progressive aging of population in economically developed countries [1]. One quarter of population of Russian Federation belongs to the category of people who are older than working age (according to Federal State Statistics Service). Such demographic situation determines the increase of the incidence of metabolic disorders; the most common of them is type 2 diabetes mellitus (DM). For the last 10 years, the world number of DM patients has been increased more than twofold and in the end of 2015, had reached 415 million. According to prognosis of the *International Diabetes Federation*, numbers of DM patients will reach about 642 million in 2040. According to data of the Federal Register, in 2016, in Russia, 4 million people were on the dispensary observation for type 2 DM that was almost 3% of the population. However, these data underestimate the real number of patients, as they consider only revealed and registered cases of disease and the real number of diabetes patients in Russia may reach 8-9 million (about 6% of the population) [2, 3].

There is a close link between DM and cardiovascular disease, which is the most prevalent cause of morbidity and mortality in diabetic patients. About 60% of patients are diagnosed with both cardiovascular disease and DM2 [4]. It is known that the risk of development of acute myocardial infarction in patients with type 2 DM is 6–10 times higher than in a whole population [5]. The combination of ischemic heart disease and diabetes is one of the most adverse conditions and leads to the significant increase of cardiovascular complications and the mortality rate.

The problem of comorbidity of these two most common noncommunicable pathologies is traditionally noted in the

Table 1: Clinical characteristics of the patients with myocardial infarction in dependence of the age.

Indicators	The 1st group ($n = 358$)	The 2nd group ($n = 504$)	p
Men/women, n (%)	158/200 (44/56)	433/71 (86/14)	<0.001
Infarction, n (%)	122 (34)	85 (17)	<0.001
Angina, n (%)	255 (71.2)	204 (40)	<0.001
Stroke, n (%)	46 (12.8)	36 (7)	0.005
Arterial hypertension, n (%)	320 (89.3)	323 (64)	<0.001
Dyslipidemia, n (%)	283 (79)	386 (76.6)	0.4
Smoker, n (%)	113 (31.6)	403 (80)	<0.001
Presence of type 2 diabetes mellitus, n (%)	92 (26)	116 (23)	0.36
Glycemic level, $\mu \pm \sigma$	$6.49 \pm 1{,}9$	$5.99 \pm 1{,}93$	0.0002
Atypical level of infarct, n (%)	15 (4.2)	64 (12.7)	<0.001
Infarction with ST elevation, n (%)	279 (78)	403 (80)	0.47

Note: $\mu \pm \sigma$: mean value and mean square deviation; p: achieved significant level.

cohort of patients of elderly and senile age. We are starting to see an increase in type 2 diabetes in leaner people at a much younger age than usually associated with the disease. So about 50% of all patients with type 2 DM in the world are 40–59 years; they are considered to be the working-age population that worsens the economic aspect of this problem [2].

Alongside with that, despite the great interest of the scientific medical community to the problem of comorbidity of ischemic heart disease and type 2 DM, the study of the long-term postinfarction prognosis of patients remains a very complex problem because of necessity to provide optimal number of patients, difficulties of overcoming artificial selectivity of the studied groups, lack of common database of persons who have suffered myocardial infarction, and so on. The use of population registers, such as the Register of Acute Myocardial Infarction, is the most optimal to study the prognostic value of DM in the long-term postinfarction period, because it can ensure the greatest objectivity of the obtained results [6].

In connection with the abovementioned, the aim of this study is to investigate the influence of type 2 diabetes mellitus on long-term prognosis of postinfarction patients in various age categories on the basis of the population taken from Register of Acute Myocardial Infarction.

2. Methods

This study was based on analysis of a prospective database "Register of Acute Myocardial Infarction," Tomsk (Russia). The basis of the information and analytical base of the RAMI is the coding table drawn up based on the specially designed "initial registration record," which contains all information about a patient (including the results of the interrogation, anamnesis data, medical history data and other medical documents, and the results of pathologist studies). The study included patients with acute myocardial infarction, who were admitted to the Cardiology Research Institute (Tomsk) with acute myocardial infarction within one year ($n = 862$). All the patients included signed the informed consent form; the study protocol was approved by the Local Ethics Committee of the Cardiology Research Institute, Tomsk. The clinical outcome after 5 years was analyzed. To analyze fatal cases from cardiovascular diseases, we have used results of the anatomical-pathological and forensic studies. The study included analysis of five-year mortality depending on age and history of diabetes mellitus 2.

The patients were categorized into 2 groups based on their ages: group 1—comprised patients older than working age ($n = 358$, men over 60, and women over 55 years old) and group 2—comprised employable patients ($n = 504$) (article 7 of the Federal Law from December 17, 2001 number 173-FZ "on labor pensions in the Russian Federation"). The main characteristics of the patients are given in Table 1.

At the admission to the hospital, 208 of the patients had the history of DM2. The diagnosis of diabetes was confirmed according to criteria of the World Health Organization. During the follow-up study, 45 more patients in the second group were found to have DM2 and 33 patients presented the onset of DM2 after discharge from the hospital. Groups were further divided into subgroups according to the duration of diabetes. Patients with diabetes received optimally matched hypoglycemic therapy including glibenclamide, gliclazide, and metformin.

A total of 208 patients were diagnosed with both cardiovascular disease and DM2.

Statistical analyses were performed with Statistica V10.0 (company "StatSoft Inc." statistical package). Categorical variables were described as frequencies and percentages. Continuous variables were described as mean ± standard deviation (SD). Verification of the quantitative data distribution has been performed using the Shapiro-Wilk test. Qualitative values are presented as absolute and relative values (n (%)). To compare quantitative data obeying the normal distribution law in two independent groups, we used Student's t-test (homogeneity of general variances has been assessed using the Levene's test). We utilized χ^2 analyses for group comparisons of each of the categorical measurements and the Fisher's test for 2 independent groups for the continuous measurements. The survival analysis in the studied groups was carried out using the Kaplan-Meier method, the comparison of the two curves was performed

using the Logrank test. To reveal factors that affect the course and prognosis of the disease, the odds ratio was calculated. The Bonferroni correction was used for multiple comparison correction when several dependent or independent statistical tests were being performed simultaneously. p values of 0.05 from two-sided tests were considered to indicate statistical significance.

3. Results

3.1. Clinical and Anamnestic Characteristics of Patients. In the first group of patients with acute myocardial infarction, there were 158 men older than 60 and 200 women older than 55. In the second group, there were 433 men and 71 women. The groups were divided according to the age of the patients, and they turned out to be different in a number of clinical and anamnestic characteristics.

The older patients suffered from arterial hypertension and angina more often. Every third patient in group 1 had history of myocardial infarction; 12.8% of patients older than working age had stroke history. The majority of patients of working age who suffered myocardial infarction were smokers. The incidence of dyslipidemia in both groups was comparable, and it was 79% and 76.6% of cases.

Every fourth patient in both groups suffered from DM2: 1st group—92 patients (26%) and 2nd group—116 patients (23%) (Table 1). Fasting blood sugar levels in patients of older age were significantly higher than in the group of working-age patients (Table 1).

3.2. Survival Rates of Patients after Myocardial Infarction Depending on Age and Diabetes Mellitus. The average 5-year mortality rate was 33.8% in group 1 and 26.8% in group 2 ($p = 0.026$). Elderly patients with DM2 had worse prognosis and increased five-year mortality compared with patients of the same age group without DM2 (OD 2.2; 95% CI 1.47–3.4; $p = 0.002$) (Figure 1).

Significant influence of the fact of the presence of type 2 DM on a long-term postinfarction prognosis of the patients has not been revealed in performing similar analysis of survival time of Kaplan-Meier in the cohort of working patients. Thus, the level of 5-year mortality among patients with type 2 DM in this group was 25% (29 persons have died during follow-up) and was comparable with the mortality rate in patients without pathology of carbohydrate metabolism that was 27.3% (106 persons have died). Time of death after myocardial infarction also did not differ between the groups of working patients with DM and patients without DM, which is evidently demonstrated by the Kaplan-Meier curves (Figure 2).

3.3. Survival Rates of Patients after Myocardial Infarction Depending on the Diabetes Mellitus Duration. In our study the comorbidities of type 2 DM and myocardial infarction (MI) in patients of working age did not show significant impact on long-term prognosis. For more detailed analysis, the group of working-age patients with both myocardial infarction and type 2 DM was assigned into 3 subgroups according to DM duration: the first subgroup included the patients that had longer duration of type 2 DM than cardiovascular disease, the second subgroup included patients with equal duration of both type 2 DM and cardiovascular disease, and the third subgroup included the patients with diabetes

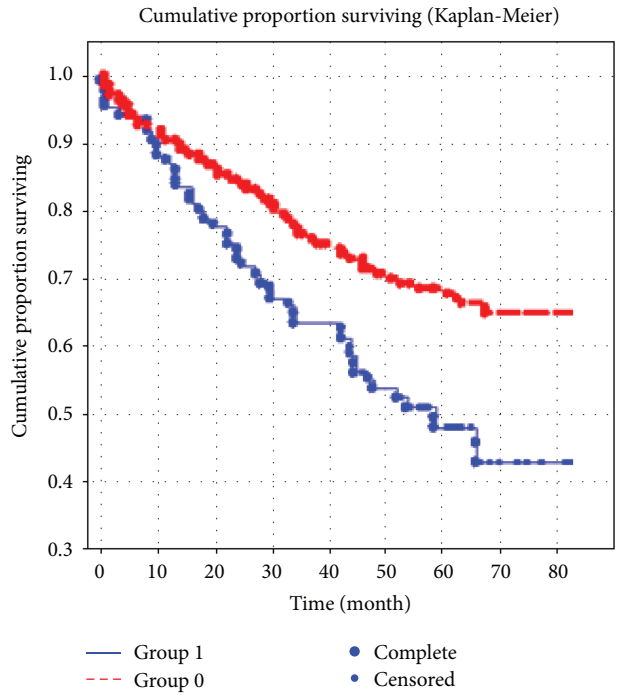

Figure 1: Impact of diabetes mellitus on long-term prognosis of the disease in elderly patients who suffered myocardial infarction. Note: presence of diabetes mellitus: 0 = no; 1 = yes.

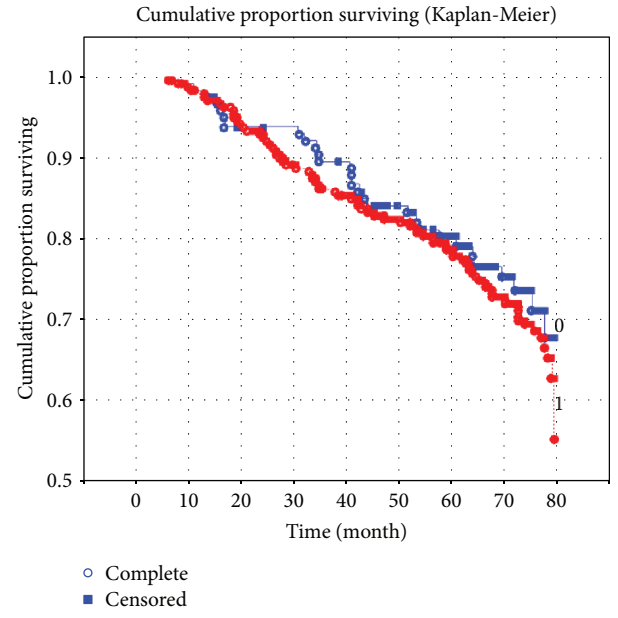

Figure 2: Influence of the presence of diabetes mellitus on long-term prognosis of the disease in patients of working age with myocardial infarction. Note: presence of diabetes mellitus: 0 = no; 1 = yes.

diagnosed during the five-year prospective study. Statistically significant differences in long-term outcomes were found in younger patients ($p = 0.004$) only in the group with longer duration of diabetes, unlike the group with DM2 onset ($p = 0.004$) (Figure 3).

4. Discussion

The results of the study showed that patients of the 1st group have significantly more serious complications due to their age, such as hypertension, angina, myocardial infarction(s), and stroke(s), which indicates the progression of existing diseases with age. At the same time, the disorder of lipid metabolism occurred with the same rate in both adult and older patients. Probably this indicator depends on genetic predisposition to the violation of lipid metabolism. In addition, the incidence of diabetes mellitus in both groups was the same at the time of inclusion of the patients into the study despite the fact that the duration of coronary artery disease was longer in the older patients. Possibly younger patients nowadays have lower physical activity and more refined food that contribute to the development of diabetes at younger age.

Our data suggest the generally accepted and proven hypothesis that type 2 diabetes mellitus increases the risk for adverse outcomes in elderly patients with MI. At the same time, we found that type 2 DM impact on long-term prognosis in patients of working age with MI strongly depends on duration of diabetes mellitus. In numerous studies, diabetes mellitus or hyperglycemia was shown to be an independent risk factor in both short- and long-term mortalities after acute MI [7]. Moreover, in the cohort study (VALIANT) diabetes mellitus was found to be an independent predictor of mortality and adverse cardiovascular events in patients of the first year after myocardial infarction. Analysis of these literature data has showed that the age of patients included in the studies was 60 years and older that corresponds to the patients of group 1 in our study which were older than the working age. Mechanisms of adverse outcomes among DM patients are related to impaired metabolic processes, including insufficiency of energy consumption of myocardium, activation of free radical processes, endothelial dysfunction, arterial thrombus formation, and fibrinolysis [8]. The DM patients are more vulnerable to development of atherosclerosis that leads to diffuse and multivascular damages of the coronary arteries. In addition, according to the literature data, diabetic cardiomyopathy can lead to the development of more severe forms of heart failure [8].

However, our study demonstrated that in the younger age group of the patients, the DM does not worsen prognosis of the survival rate but the survival rate depends on the duration of diabetes mellitus. So, the patients with DM developed after onset of myocardial infarction have better outcomes than the patients with DM diagnosed before or at the same time with myocardial infarction. But at the same time, some experimental studies show paradoxical increase of heart stability to damaging actions of ischemia and reperfusion in animals with DM [9, 10]. In previously published articles, we have also demonstrated that in inducing the onset of

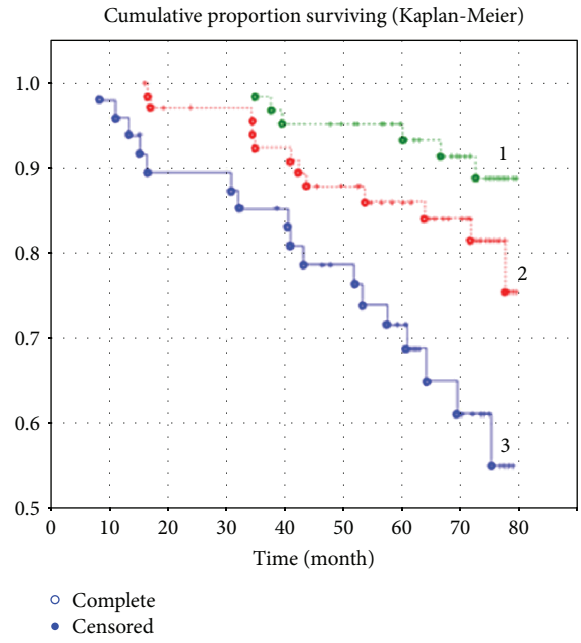

Figure 3: Survival rate of patients of working age with acute myocardial infarction according to duration of type 2 diabetes mellitus. Note: 1 = DM2 diagnosed in the postinfarction period ($n = 38$); 2 = DM2 diagnosed at the admission ($n = 45$); 3 = patients who had history of DM2 before the infarction occurred ($n = 38$).

DM in rats in 2 weeks after myocardial infarction stimulation, the contractile function of isolated papillary muscle remains the same like in intact animals and the level of calcium-transporting proteins of sarcoplasmic reticulum of cardiomyocytes is also comparable with the one in healthy myocardium [11]. Moreover, we revealed that isolated trabeculae of the heart of ischemic heart disease (IHD) patients with short duration of DM maintained positive rhythm inotropic response of myocardium that correlated with a higher level of Ca^{2+}-ATPase of sarcoplasmic reticulum (SR) in comparison with the same parameters of myocardium in IHD patients without DM [12]. Since Ca^{2+}-ATPase of SR is energy-dependent enzyme, the efficiency of its work depends on not only the amount of enzyme but also on the availability of energy substrate. It has been shown that ATP produced in the glycolysis process is an essential source of energy for membrane transport of Ca^{2+}, in particular for Ca^{2+}-ATPase ion pump of SR. In chronic ischemia, ATP synthesis in cardiomyocytes is carried out as a result of the process of glycolysis [13]. Probably in patients with CD2 at early stage of disease development, hyperglycemia can increase the availability of the additional substrates for glycolytic processes that promote normalization of energy supply of metabolic reactions in pathological cells. According to our previously published data at the combined development of postinfarction cardiosclerosis and DM in rats, cardiomyocytes use both fatty acids and glucose as energy substrate.

In accordance with our study, clinical data showing that patients with DM have the lower risk of adverse cardiovascular events in comparison with patients with ischemic heart

disease has been appeared [14, 15]. In these studies, it is noted that mortality from acute myocardial infarction in patients with DM is lower than in patients with ischemic heart disease regardless of their age (the patients included in the study were 30–80 years).

However, type 2 diabetes of more than 10-year duration or type 2 DM with severely elevated glucose levels has been noted the same predictive value on the survival rate after myocardial infarction as for the IHD patients [14, 15]. In addition, a higher level of glucose in blood plasma at admission to hospital is an adverse prognostic factor for patients without diabetes in comparison with patients with established diagnosis of DM [16]. The presented data allow us to take a new look on the problem of DM in cardiovascular pathology and on the disclosure of the mechanisms causing the increase of heart resistance to ischemic damage. It is well known that all experimental works which shown the cardioprotective effect of DM were performed on young animals. That fact corresponds to our results obtained in group 2 [9, 10, 17, 18]. However, elderly Goto-Kakizaki rats (model of type 2 DM) have increased sensitivity to ischemic heart diseases [19].

Probably in the early developing of DM and in the patients of younger age, we can see the initiation of the so-called "metabolic preconditioning." The mechanisms of this phenomenon can be connected with increase of the expression of antioxidant protection factors and enzymes of PI3K/Akt signaling pathways and with decrease of the expression of apoptosis genes, proinflammatory cytokines (TNF-α), profibrogenic transforming growth factor-β, and alpha actin-1—a hypertrophy marker [17]. Nowadays, the important role in realization of cardioprotective effects is given to protein—AMP-activated protein kinase—one of the key enzymes that initiates the process of energy saving in a cell. It was revealed that DM itself can lead to activation of this protein—AMP-kinase [20]; in addition, it was found that the expression rate of glucose transporter GLUT-1 in the cardiomyocytes of diabetic animals on 15th day after infarction was higher than the expression rate of glucose transporter in diabetic rats without ischemic damage [17]. Also, we can mention angiogenesis as a mechanism reducing the damage effects of ischemia before and after simulation of experimental myocardial infarction which is accompanied with decrease of the fibrosis processes in condition of hyperglycemia [17]. The sympathetic nervous system also plays a great role in pathogenesis of ischemic damages. It was found that while the sympathetic nervous system (SNS) tone decreases, induced by myocardial infarction in diabetic rats, the antioxidant protection of heart increases and of activity of prooxidant enzymes decreases [18]. Clinical data obtained from the study showed ambiguous prognostic effects of type 2 diabetes mellitus in patients with myocardial infarction consistent with those of experimental studies. Complex analysis of clinical and experimental data in this study area will help to develop the system of cardiovascular risk stratification in patients with both type 2 diabetes and coronary heart disease and will provide the opportunities for the personification of therapeutic approaches reducing damages of the ischemic factor effects.

5. Conclusions

The combination of both type 2 diabetes and coronary heart disease in elderly patients was showed to be a prognostically adverse factor, leading to a significant increase in the 5-year mortality rate. At the same time, younger patients with myocardial infarction did not have any significant differences in the long-term outcome according to the presence of diabetes mellitus while a longer duration of DM2 significantly worsened prognosis during the postinfarction period.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

Authors declare that they have no obvious and potential conflicts of interests connected with the publication of the present paper.

Acknowledgments

This work was supported by the Russian State Projects "Science" no. AAA-A15-115123110026-3 with respect to research topic of Cardiology Research Institute, Tomsk National Research Medical Center of the Russian Academy of Sciences.

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Hindawi
Journal of Diabetes Research
Volume 2018, Article ID 8485624, 10 pages
https://doi.org/10.1155/2018/8485624

Research Article

Exercise Training Induced Cardioprotection with Moderate Hyperglycemia versus Sedentary Intensive Glycemic Control in Type 1 Diabetic Rats

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Received 7 March 2018; Accepted 16 May 2018; Published 5 July 2018

Academic Editor: Markus Wallner

Intensive insulin therapy (IIT; 4–7 mmol/L) is the preferred treatment for type 1 diabetes mellitus (T1DM) patients to reduce the risk of cardiovascular disease (CVD). However, this treatment strategy has been questioned as it is accompanied with a sedentary lifestyle leading to weight gain and insulin resistance. T1DM patients who partake in high-intensity aerobic training (AT_{high}) to reduce CVD often utilize conventional insulin therapy (CIT; 9–15 mmol/L) to offset the risk of hypoglycemia. Moreover, exercise modalities incorporating resistance training (RT) have been shown to further reduce this risk. The purpose of this investigation was twofold: (1) to determine if CIT paired with AT_{high} results in larger cardioprotection from an ischemia-reperfusion (I-R) injury than IIT and (2) to establish if the integration of RT with AT_{high} (ART) results in similar cardioprotection as AT_{high}. Diabetic (D) male Sprague-Dawley rats were divided into D-IIT ($n = 12$), D-CIT ($n = 12$), D-AT_{high} ($n = 8$), D-RT ($n = 8$), and D-ART ($n = 8$). T1DM was induced with streptozotocin, and blood glucose was adjusted with insulin. D-AT_{high} occurred on a treadmill (27 m/min; 1 hr), D-RT performed weighted ladder climbs, and D-ART alternated daily between AT_{high} and RT. Exercise occurred 5 days/wk for 12 wks. This investigation demonstrates that cardioprotection following an I-R injury was similar between D-AT_{high} and D-IIT. This cardioprotection is not exercise-specific, and each provides unique advantages. D-AT_{high} leads to improved glycemia while insulin sensitivity was enhanced following resistance exercises. Thus, exercise is an effective means to elicit cardioprotection in T1DM. However, in addition to glycemia, other factors should be considered when tailoring an exercise program for T1DM patients.

1. Introduction

Individuals with type 1 diabetes mellitus (T1DM) exhibit a heightened risk for cardiovascular disease (CVD) not entirely accounted for by traditional risk factors (hyperglycemia, obesity, hypertension, dyslipidemia, and smoking) [1]. To date, the most characterized strategies to limit CVD development have been intensive insulin therapy (IIT) [2] and regular exercise [3–5]. However, both IIT and exercise potentiate the risk of hypoglycemia, especially when attempted collectively [2, 6]. To counteract hypoglycemia risk, individuals with T1DM often intentionally elevate their blood glucose concentrations prior to exercise through changes in insulin dosing and/or carbohydrate ingestion [7]. As such, individuals with T1DM who are more physically active typically prescribe to a more conventional insulin therapy (CIT) and have higher HbA1c values with reduced focus on glycemic control [8].

Additional work is needed to better evaluate the cardiovascular benefits and risks associated with regular exercise in physically active individuals with T1DM that often prescribe to less stringent glycemic control, since elevations in glycemia (HbA_{1c}) are known to increase the risk of cardiovascular complications [2]. Our group has demonstrated that

the combination of less stringent blood glucose control and high-intensity aerobic exercise training (AT_{high}) in experimental T1DM rats not only decreases the risk of exercise-induced hypoglycemia [4] but also has numerous cardiovascular benefits such as increased recovery from an ischemic insult [4], reduction in cardiovascular autonomic dysfunction [9], improvement in systolic and diastolic heart function [5], and improved vascular reactivity [10, 11]. It is unknown how these cardiovascular benefits would compare to stringent blood glucose control alone (i.e., IIT), the predominant treatment option for individuals with T1DM [2, 12]. This is a significant question that needs to be answered since IIT is associated with cardiovascular risk factors such as increased sedentary behaviour, weight gain, and insulin resistance [8, 13, 14].

Additionally, it has been established both experimentally [15] and clinically [16] that poor glycemic control leads to hepatic glycogen deficiencies. Restoration of hepatic glycogen content could represent a mechanism for combatting hypoglycemia, as hepatic glycogen is the predominant source of blood glucose during exercise [17] and insulin overcorrection [18]. Our laboratory has recently shown that ten weeks of AT_{high} fails to normalize hepatic glycogen in T1DM rats despite significantly elevated levels of hepatic glycogenic storage enzymes [15]. In contrast, resistance training (RT) has been shown to increase hepatic glycogen content in rats [19], while also alleviating the risk of exercise-induced hypoglycemia in T1DM [4, 20]. While still allowing for the cardiovascular benefits associated with regular aerobic exercise, the integration of RT with aerobic exercise may allow individuals with T1DM to exercise safely by reducing the risk of hypoglycemia development. Indeed, the Canadian Diabetes Association recommends that RT be incorporated into aerobic exercise regimes at least twice a week [21].

The objective of the present study was to examine whether moderate blood glucose control and AT_{high} result in greater levels of cardioprotection than more stringent blood glucose control. Secondly, it was determined whether combining RT with AT_{high} resulted in similar cardioprotection and less exercise-induced blood glucose fluctuations. Additionally, the potential relationship between glycemic status and cardioprotection was explored.

2. Methods

This study was approved by the Research Ethics Board of the University of Western Ontario which is in compliance with the guidelines of the Canadian Council on Animal Care. Eight-week-old male Sprague-Dawley rats were obtained from Charles River Laboratories, provided standard rat chow ad libitum, and housed in pairs at a standard temperature and humidity (21.5°C and 50% humidity).

2.1. Experimental Protocol. Sprague-Dawley rats were randomly divided into one of five diabetic groups (D): conventional insulin therapy (D-CIT; $n = 12$), intensive insulin therapy (D-IIT; $n = 12$), high-intensity aerobic exercise training (D-AT_{high}; $n = 8$), resistance exercise training (D-RT; $n = 8$), and combination aerobic/resistance exercise training (D-ART; $n = 8$). During experimental week one, T1DM was induced after five consecutive daily injections of streptozotocin (Sigma-Aldrich; 20 mg/kg; dissolved in 0.1 M citrate buffer, pH 4.5) and T1DM was confirmed after two nonfasting blood glucose concentrations greater than 18 mmol/L. After diabetes confirmation, subcutaneous insulin pellets (Linshin, Toronto, Canada) were implanted in the abdomen (experimental week two). Through insulin pellet adjustments, it was intended to maintain blood glucose concentrations in D-CIT, D-AT_{high}, D-RT, and D-ART between 9 and 15 mmol/L and D-IIT between 4 and 9 mmol/L. Exercise training occurred five times a week over a twelve-week period (experimental week 3 to 14). D-AT_{high} rats exercised on a motorized treadmill at 27 m/min (six percent grade) for one hour. Continuous running was encouraged by small blasts of compressed air at the rear of the treadmill. In D-RT rats, resistance training consisted of climbing a vertical ladder with weights secured to the proximal portion of the tail, as previously described [4]. Familiarization occurred the week prior to training (experimental week 2) and consisted of 10 climbs a day with varying weights attached (5%, 15%, 20%, and 35% of each rat's body mass). Regular resistance training sessions (experimental week 3 to 14) consisted of incremental increases in weight (50%, 75%, and 90% of maximal lifting capacity) followed by 100% of their maximal lifting capacity until exhaustion (unable to finish climb despite tactile stimulation to haunches). Maximum lifting capacity was calculated every fourth exercise session and was determined by sequentially adding 30 grams of weight to the rat's tail until exhaustion (starting at 75% of their body mass). In D-ART rats, exercise training consisted of alternating daily between the aerobic and resistance exercises.

2.2. Blood Analysis. Blood samples were taken over two consecutive days from the saphenous vein during the last week of exercise training (experimental week 14; pre/postexercise) to determine if antecedent AT_{high} or RT altered the blood glucose response to a subsequent exercise bout [22]. In D-ART, this measure was conducted at week 11 and week 12 of training (experimental week 13 and 14, resp.) to determine if performing AT_{high} (or RT) first had an effect on glucoregulation following a subsequent bout of RT (or AT_{high}). Blood glucose concentrations were detected using a OneTouch Ultra 2 Blood Glucose Monitoring System (Lifescan Canada Ltd., Burnaby, BC, Canada) and OneTouch test strips (Lifescan Canada Ltd.). Epinephrine concentrations prior to and after exercise were determined via ELISA (Cusabio, catalog number CSB-E08678r). Fructosamine concentrations were determined using the procedure outlined by Oppel et al. [23]. Briefly, serum samples taken at the completion of the study were added to a carbonate buffer (pH 10.8) containing 0.25 mM nitroblue tetrazolium (NBT) at 37°C. Following a 20-minute incubation at 37°C, the reaction was read at 530 nm and compared to standards of 1-deoxy,1-morpholinofructose (DMF; Sigma-Aldrich) and albumin (40 g/L).

2.3. Langendorf Heart Preparation. Three days following the last exercise bout, all rats were anaesthetized with isoflurane and hearts were extracted and placed in cold

Table 1: General animal characteristics at the completion of the study.

	D-CIT	D-IIT	D-AT$_{high}$	D-RT	D-ART
Body mass (g)	567 ± 20	598 ± 21[3,4,5]	510 ± 15	520 ± 21	534 ± 19
Blood glucose conc. (mmol/L)	15.0 ± 1.2	10.9 ± 1.2[1,3,5]	15.6 ± 0.5	12.4 ± 1.9[5]	16.7 ± 1.4
Fructosamine conc. (mmol/L)	3.0 ± 0.5	1.0 ± 0.2[1,5]	1.3 ± 0.3[1,5]	2.0 ± 0.1	2.6 ± 0.7
Exogenous insulin (IU)	27.3 ± 5.4	35.8 ± 7.8	19.0 ± 7.7	11.1 ± 7.1[2]	4.1 ± 2.0[1,2]
Insulin resistance (AU)	10,665 ± 2078[5]	16,055 ± 4558[3,4,5]	4722 ± 1988	1438 ± 62[3]	1260 ± 601[3]

Data are means ± SE. [1]Different from D-CIT; [2]different from D-IIT; [3]different from D-AT$_{high}$; [4]different from D-RT; [5]different from D-ART.

Krebs-Henseleit buffer (KHB; 120 mM NaCl, 4.63 mM KCl, 1.17 mM KH_2PO_4, 1.25 mM $CaCl_2$, 1.2 mM $MgCl_2$, 20 mM $NaHCO_3$, and 8 mmol/L glucose). Hearts were rapidly cannulated for unpaced retrograde perfusion of KHB (37°C; gassed with 95% O_2 and 5% CO_2) at 15 mL/min. A small water-filled latex balloon was inserted through the mitral valve and into the left ventricle. Hearts were equilibrated to the preparation for 30 minutes (preischemia) followed by the termination of flow for 50 minutes. Subsequently, reperfusion occurred for a total of 30 minutes at 15 m/min. Left ventricle pressures (LVDP, left ventricle developed pressure; LVEDP, left ventricle end-diastolic pressure) were measured with a pressure transducer (Statham Gould P23ID), and the rate of pressure development (+dp/dt) and relaxation (−dp/dt) were obtained using a PowerLab 8/30 data acquisition system and analyzed by LabChart 7.0 Pro software (ADInstruments, Colorado Springs, Colorado, USA). Area under the curve (AUC) was determined for the pressure curves of each rat in the study in order to correlate measures to glycemic control and insulin resistance.

2.4. Glucose Tolerance Test. Intravenous glucose tolerance tests (IVGTT) were conducted following training (experimental week 14) after an 8–12-hour fast and consisted of a sterile injection (1 g/kg) of dextrose solution (50% dextrose, 50% ddH_2O) into the lateral tail vein. Blood glucose concentrations were measured at 5, 10, 20, 30, and 40 minutes postinjection, and area under the curves (AUC) were determined for each individual rat. Prior to the IVGTT, blood samples were taken from the saphenous vein and exogenous insulin concentrations were measured via ELISA (Alpco, Salem, NH: catalog number 80-INSHU-E01.1). The measure of insulin resistance was considered the AUC of the IVGTT multiplied by exogenous insulin concentration. We have previously reported that when using this T1DM model, sedentary rats can become insulin resistant and require substantial more exogenous insulin in order to maintain the desired blood glucose concentrations [15, 24]. Accordingly, when determining the insulin resistance measure, the amount of circulating insulin present in the rat during the IVGTT was factored into the calculation.

2.5. Western Blotting. Liver (extracted during sacrifice at end of study) and left ventricles were homogenized in buffer (100 mM NaCl, 50 mM Tris base, 0.1 mM EDTA, and 0.1 EGTA, pH ~7.5) using a polytron, and total protein concentrations were determined by the Bradford protein assay. Homogenates (40–80 μg of protein) were mixed with equal volumes of sample buffer (0.125 M Tris, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.015% bromophenol blue, pH ~6.8), separated by SDS-PAGE (4% stacking, 10% separating) and transferred to nitrocellulose membranes. Membranes were blocked in 5% nonfat dairy milk in TTBS (10 mM Tris, 100 mM NaCl, and 0.1% Tween-20, pH 7.5) for 1 hour and incubated overnight at 4°C with primary antibodies (Cell Signaling: Hsp70 1 : 4000, glycogen synthase 1 : 1000; Abcam: glycogen phosphorylase 1 : 2000, SERCA2 1 : 1000; Santa Cruz: glucose-6-phosphotase 1 : 200) diluted in TTBS with 2% nonfat dairy milk. Following washes in TTBS, membranes were exposed to corresponding secondary antibodies (IgG-HRP conjugated, Bio-Rad) in TTBS with 2% nonfat dairy milk for 1 hour at room temperature. After successive washes in TTBS, protein bands were visualized with a luminol-based chemiluminescent substrate (Western C Enhanced Chemiluminescent Kit; Bio-Rad), imaged with the Chemidoc XRS System (Bio-rad), and analyzed with Quantity One Software (Bio-Rad). Optical densities were normalized to a consistent non-T1DM control sample and subsequently β-actin.

2.6. Statistical Analysis. Body mass, blood glucose, fructosamine, exogenous insulin, insulin resistance, and Western blot data were compared using a one-way analysis of variance (ANOVA). Langendorf measures were compared using a two-way repeated measure ANOVA. Blood glucose concentrations and epinephrine concentrations in response to exercise, and over consecutive days, were compared using a two-way repeated measures ANOVA. When a significant difference was found, a least squares difference post hoc test was performed and significance was set at $p < 0.05$. Relationships between left ventricular mechanical performance and fructosamine or insulin resistance were determined via Pearson correlation. All data are presented as a mean ± standard error. All statistical analyses were completed using GraphPad Prism 6.

3. Results

3.1. Animal Characteristics. Blood glucose concentrations were lower in D-IIT compared to D-CIT (p=0.03), D-AT$_{high}$ ($p = 0.02$), and D-ART ($p = 0.007$), and lower in D-RT compared to D-ART ($p = 0.04$) (Table 1). Body mass was higher in D-IIT compared to D-AT$_{high}$ ($p = 0.003$), D-RT ($p = 0.01$), and D-ART ($p < 0.04$). Fructosamine concentrations were lower in D-IIT compared to D-CIT ($p < 0.001$) and D-ART ($p = 0.01$). Further, fructosamine concentrations

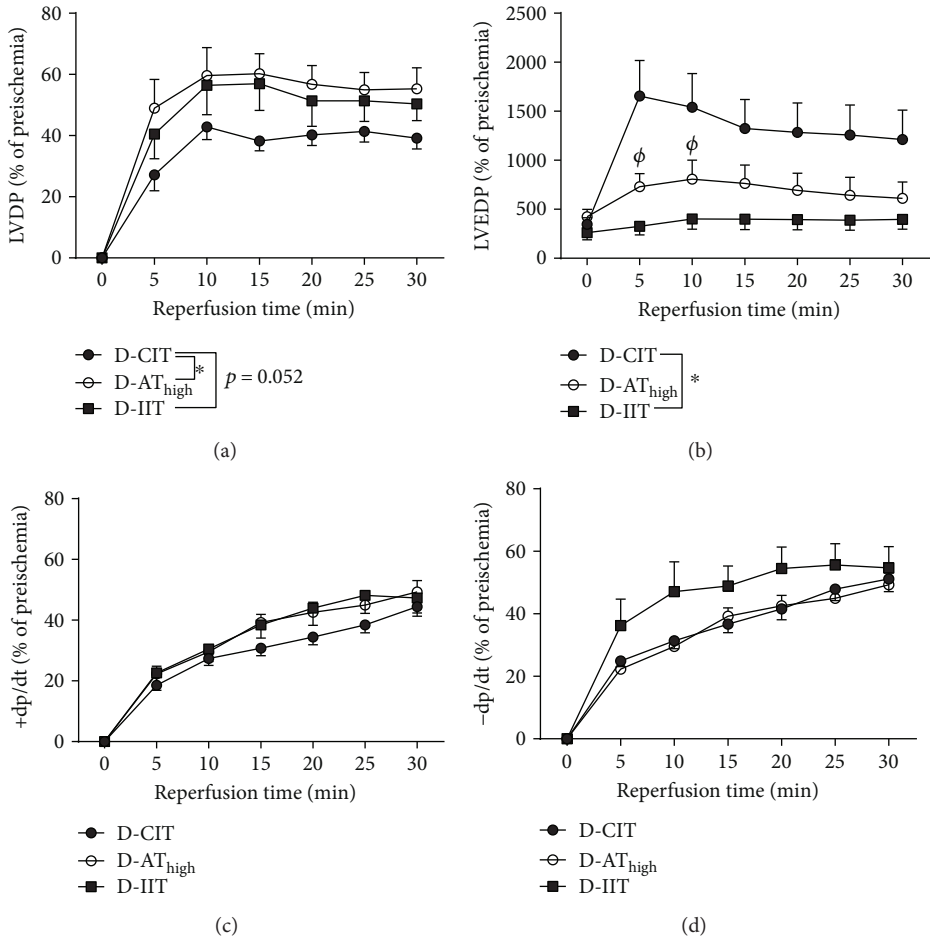

Figure 1: Left ventricle mechanical performance during ischemia-reperfusion. The data are presented in time course format. LVDP (a), LVEDP (b), +dP/dt (c), −dP/dt (d). *Significant main effect ($p < 0.05$); (φ) different from D-CIT ($p < 0.05$). Data are presented as a mean ± SE.

were lower in D-AT$_{high}$ compared to D-CIT ($p = 0.003$) and D-ART ($p = 0.04$). Exogenous insulin concentrations were lower in D-RT compared to D-IIT ($p = 0.01$), and lower in D-ART compared to D-CIT ($p = 0.02$) and D-IIT ($p < 0.001$). The insulin resistance measure was higher in D-IIT compared to D-AT$_{high}$ ($p = 0.02$), D-RT ($p = 0.008$), and D-ART ($p = 0.003$). Further, D-CIT displayed higher insulin resistance compared to D-ART ($p = 0.04$).

3.2. Left Ventricular Mechanical Performance. For the first objective of the study, we compared left ventricular mechanical performance following ischemia in D-CIT, D-AT$_{high}$, and D-IIT. There was a significant increase in LVDP in D-AT$_{high}$ compared to D-CIT (Figure 1(a); $p = 0.03$). No difference in LVDP was observed between D-AT$_{high}$ and D-IIT ($p = 0.5$), and LVDP between D-CIT and D-IIT did not reach significance ($p = 0.052$). LVEDP was lower in D-IIT compared to D-CIT (Figure 4.1B; $p = 0.004$), while D-AT$_{high}$ was lower than D-CIT at 5 ($p < 0.0001$) and 10 minutes ($p = 0.03$) during reperfusion. There was no difference in +dp/dt ($p = 0.4$) or −dp/dt ($p = 0.2$) across any of the groups (Figures 1(c) and 1(d)).

For the second objective of the study, we compared left ventricle mechanical performance following ischemia in D-AT$_{high}$, D-RT, and D-ART. There were no differences in LVDP ($p = 0.6$) or LVEDP ($p = 0.9$) among D-AT$_{high}$, D-RT, and D-ART (Figures 2(a) and 2(b)). Compared to D-AT$_{high}$, D-ART had a higher +dp/dt at 25 ($p = 0.01$) and 30 minutes ($p = 0.005$) during reperfusion (Figure 2(c)). Compared to D-RT, D-ART had a higher +dp/dt at 25 ($p = 0.009$) and 30 minutes ($p = 0.002$) during reperfusion. D-AT$_{high}$ had a slower −dp/dt than D-RT at 20 ($p = 0.04$), 25 ($p = 0.001$), and 30 minutes ($p < 0.0001$) (Figure 2(d)). Further, D-AT$_{high}$ had a slower −dp/dt than D-ART at 20 ($p = 0.04$), 25 ($p = 0.002$), and 30 minutes ($p < 0.0001$).

3.3. Correlations of Left Ventricular Mechanical Performance. There was a significant correlation between the AUC of LVDP and fructosamine concentration (Table 2; $p = 0.01$; $r = -0.4$), while no correlation was evident between the AUC of LVEDP ($p = 0.8$), +dp/dt ($p = 0.7$), −dp/dt ($p = 0.8$), and fructosamine concentration. There was a significant correlation between the AUC of +dp/dt and insulin resistance ($p = 0.03$; $r = -0.4$), but no correlation between insulin resistance and the AUC of LVDP ($p = 0.7$), LVEDP ($p = 0.65$), or −dp/dt ($p = 0.5$).

3.4. Molecular Analysis. An elevation in left ventricle Hsp70 content was evident in D-AT$_{high}$ compared to both D-CIT

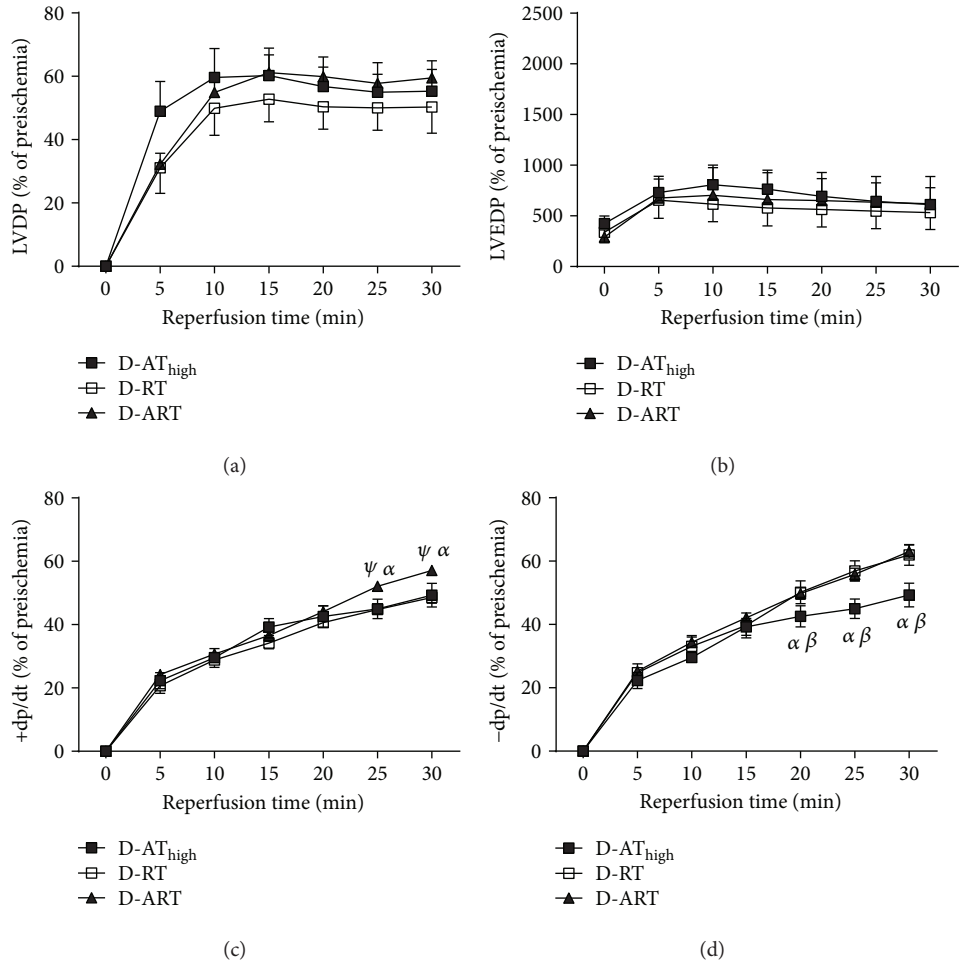

Figure 2: Left ventricle mechanical performance during ischemia-reperfusion and following different modalities of exercise training. The data are presented in time course format. LVDP (a), LVEDP (b), +dP/dt (c), −dP/dt (d). ψ: different from D-AT$_{high}$ ($p < 0.05$); α: different from D-RT ($p < 0.05$); β: different from D-ART ($p < 0.05$). Data are presented as a mean ± SE.

Table 2: Correlation of left ventricle mechanical performance on glycemia and insulin resistance.

	Versus fructosamine (mmol/L)		Versus insulin resistance (AU)	
	p value	r	p value	r
LVDP (AUC)	*0.01**	−0.4	0.7	—
LVEDP (AUC)	0.8	—	0.7	—
+dp/dt (AUC)	0.7	—	*0.03**	−0.4
−dp/dt (AUC)	0.8	—	0.5	—

*Significant ($p < 0.05$).

($p = 0.003$) and D-IIT ($p = 0.009$) (Figure 3(a)), while no differences were evident in SERCA2 among D-CIT, D-IIT, and D-AT$_{high}$ (Figure 3(b); $p = 0.8$). Differences existed between exercise regimes in that D-AT$_{high}$ resulted in higher left ventricle Hsp70 compared to D-RT ($p = 0.02$), but did not differ significantly from D-ART (Figure 3(a); $p = 0.1$). No differences in SERCA2 expression were evident among exercise regimes (Figure 4.3B; $p = 0.4$).

3.5. Hepatic Glycogen Content and Regulatory Enzymes. Hepatic glycogen content was higher in D-CIT compared to D-AT$_{high}$ ($p = 0.05$), D-RT ($p = 0.01$), and D-ART ($p = 0.004$) (Figure 4(a)). Hepatic glycogen was also higher in D-IIT compared to D-AT$_{high}$ ($p = 0.04$), D-RT ($p = 0.01$), and D-ART ($p = 0.004$). No differences in glycogen synthase ($p = 0.9$), glycogen phosphorylase ($p = 0.9$), and glycogen-6-phosphatase ($p = 0.7$) were apparent between experimental groups (Figures 4(b)–4(d)).

3.6. Exercise-Mediated Changes in Blood Glucose. Significant declines in blood glucose concentrations following exercise were apparent in D-AT$_{high}$ at day 1 and day 2 of exercise (week 12 of training; Table 3; $p < 0.0001$). No change in blood glucose concentrations was apparent following RT at day 1 or day 2 (week 12 of training; $p = 0.5$). In D-ART, significant declines in blood glucose concentrations were apparent only on AT$_{high}$ days at both week 11 ($p = 0.0003$) and week 12 ($p < 0.0001$) of training. In D-AT$_{high}$, no change in epinephrine concentrations was evident from pre to postexercise (week 12 of training; $p = 0.4$; Table 4), and epinephrine concentrations were similar between day 1 and day 2 (week 12 of training; $p = 0.2$). In D-RT, no change in

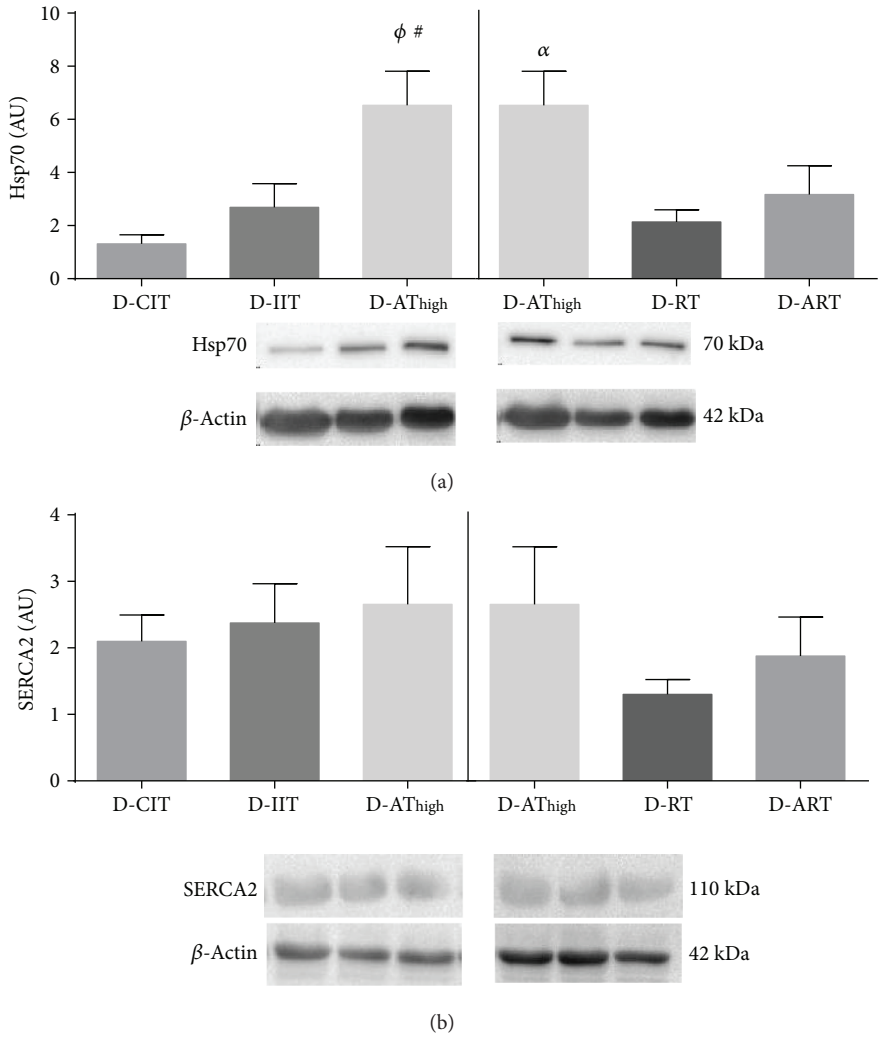

Figure 3: Left ventricle Hsp70 (a) and SERCA2 (b) protein content. ϕ: different from D-CIT; #: different from D-IIT; α: different from D-RT. Significance $p < 0.05$. Data are presented as a mean ± SE.

epinephrine concentrations was evident from pre to postexercise (week 12 of training; $p = 0.7$); however, epinephrine concentrations were lower overall on day 2 compared to day 1 (week 12 of training; $p = 0.02$). In D-ART, when RT occurred the day before AT_{high} (week 11 of training), epinephrine concentrations were reduced overall during AT_{high} ($p = 0.007$). In D-ART, no change in epinephrine occurred from pre to postexercise at both week 11 ($p = 0.5$) and week 12 ($p = 0.2$) of training.

4. Discussion

Stringent management of blood glucose concentrations through intensive insulin therapy is the primary treatment strategy in order to limit the progression of CVD in patients with T1DM [2]. Indeed, D-IIT resulted in greater recovery from an I-R injury than D-CIT, supporting the deleterious effects of chronic hyperglycemia on the macrovasculature in individuals with T1DM [12, 25]. In a previous study, we reported that six weeks of high-intensity aerobic exercise led to significant improvements in I-R functional recovery [4]. Here, we demonstrate that this modality of exercise when combined with CIT can lead to comparable recovery from an I-R injury as IIT alone. It is important to note that while exercised animals were maintained in a chronic hyperglycemic state, AT_{high} exhibited similar serum fructosamine concentrations as IIT. It is likely that glycemic control played a significant role in contributing to the increased cardioprotection of IIT and AT_{high}, since both exhibited similar serum fructosamine concentrations. Indeed, the negative correlation between serum fructosamine, indicative of glycemic control, and LVDP would support this finding.

While it is well-recognized that regular exercise can improve glycemic control (lowered HbA_{1C}) in type 2 diabetes, results in T1DM have generally failed to show this glycemic benefit [3]. A number of factors may contribute to this lack of evidence in previous studies, including the predominant use of adolescent subjects, the use of questionnaires to estimate activity levels, or the increased food consumption that is typically associated with the initiation of an exercise program [3]. Although comparable to HbA_{1c}, the measure of glycosylated hemoglobin, fructosamine is a measure of

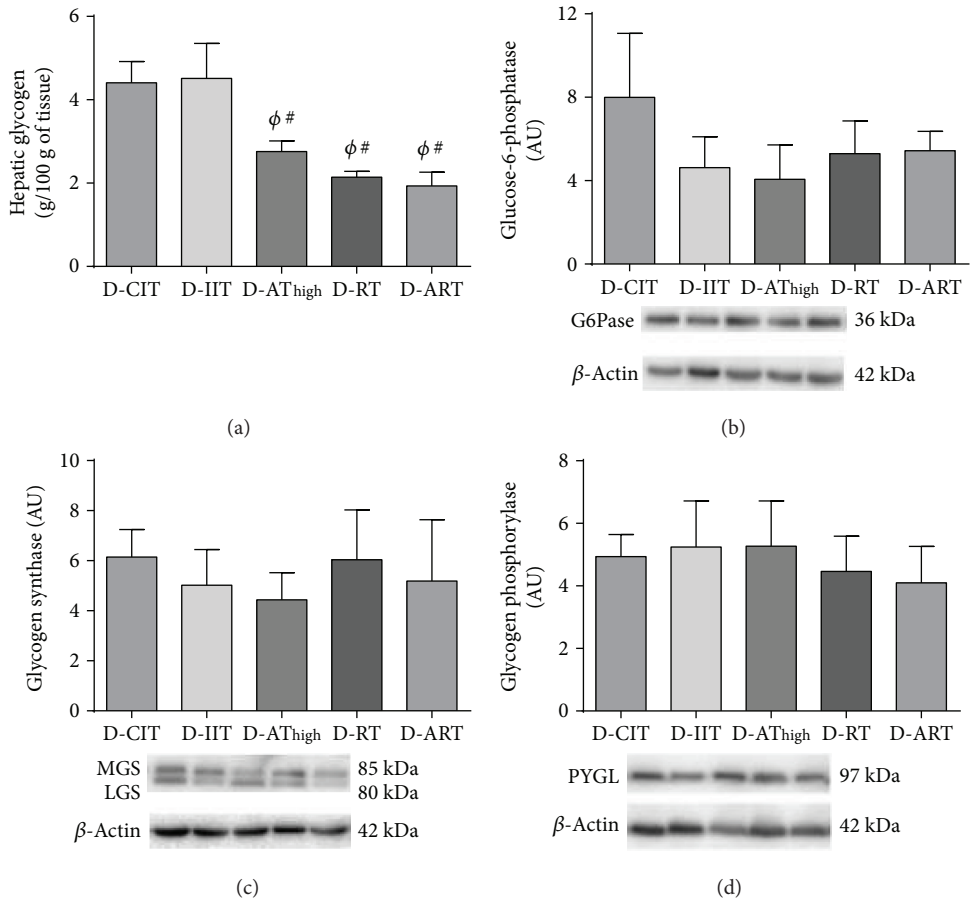

Figure 4: Hepatic glycogen content (a), glycogen-6-phosphatase (b), glycogen synthase (MGS: muscle glycogen synthase; LGS: liver glycogen synthase) (c), and glycogen phosphorylase (d). ϕ: different from D-CIT ($p < 0.05$); #: different from D-IIT ($p < 0.05$). Data are presented as a mean ± SE.

Table 3: Blood glucose concentrations in response to exercise at week 11 or week 12 of training.

	Day 1		Day 2	
	Preexercise (mmol/L)	Postexercise (mmol/L)	Preexercise (mmol/L)	Postexercise (mmol/L)
D-AT_{high}	15.0 ± 0.4	8.0 ± 1.1*	14.6 ± 0.5	6.9 ± 1.0*
D-RT	12.2 ± 1.6	12.0 ± 0.9	13.6 ± 2.2	15.1 ± 1.6
D-ART (week 11; RT then AT_{high})	14.9 ± 1.6	15.6 ± 1.2	15.4 ± 1.7	8.0 ± 1.8*
D-ART (week 12; AT_{high} then RT)	16.7 ± 1.4	8.8 ± 1.4*	15.2 ± 2.0	15.6 ± 1.2

Data are means ± SE. *Significantly lower than preexercise ($p < 0.05$).

Table 4: Epinephrine concentrations in response to exercise at week 11 or week 12 of training.

	Day 1		Day 2	
	Preexercise (pg/mL)	Postexercise (pg/mL)	Preexercise (pg/mL)	Postexercise (pg/mL)
D-AT_{high}	254.3 ± 44.6	93.1 ± 24.9	237.0 ± 43.8	298.0 ± 109.1
D-RT	320.8 ± 72.2	238.3 ± 58.2	110.0 ± 46.1*	136.3 ± 66.3*
D-ART (week 11; RT then AT_{high})	254.3 ± 107.2	150.8 ± 98.0	38.7 ± 9.3*	57.8 ± 15.1*
D-ART (week 12; AT_{high} then RT)	331.0 ± 169.8	112.6 ± 45.7	202.2 ± 33.0	184.6 ± 41.8

Data are means ± SE. *Significantly lower than day 1 ($p < 0.05$).

the amount of serum proteins that have undergone glycation and is thus a better marker for shorter-term glycemic control (approximately two weeks). While there is a shortage of evidence supporting increased glycemic control in T1DM following aerobic exercise [3], exercise intensity appears to play a significant role as to whether glycemic benefits are

obtained [3, 26]. In the present study, the aerobic exercise training program was intensive, representing approximately 70–80% of the rats VO_{2max} [27]. The potential ability of RT to improve glycemic control (determined by HbA_{1C}) in populations with T1DM is inconclusive [28], and the present results would support work citing that it has no benefit on long-term glycemia [29]. There was no improvement in fructosamine levels in D-ART, despite supplementing RT with AT_{high}, suggesting that the frequency of AT_{high} may be an important factor to experience glycemic benefits. However, since food consumption was not directly measured in the current study, it cannot be completely discounted that the diets of experimental groups may not have been isocaloric.

In a previous report, we demonstrated that six weeks of RT provided little protection against an I-R injury in T1DM rats [4]. The current study demonstrated that longer term RT, conducted alone or paired with AT_{high} (D-ART), is necessary in order to provide similar levels of cardioprotection as performing strictly AT_{high}. Indeed, it has been demonstrated in non-T1DM rats that short-term RT provides little cardioprotection [30]; however, if the RT is prolonged, the cardioprotective effects of this form of exercise become evident, as demonstrated by reduced infarct size following an I-R injury [31]. Again, it is of importance to note that we see unique advantages associated with RT that were not evident in other modalities of exercise. The maximal rate of pressure development (+dp/dt) and relaxation (−dp/dt) in T1DM rats were significantly improved in experimental groups utilizing RT that were not evident in D-AT_{high}. It has been reported that just a single bout of RT can improve the rate of left ventricular systolic pressure in hypertensive rats undergoing Langendorf perfusion [32]. Further, Melo et al. [33] reported faster cardiomyocyte contraction and relaxation in rats following eight weeks of RT, believed to be due to increased sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) expression. While neither +dp/dt nor −dp/dt were altered in D-AT_{high}, these results may not be surprising as little change in Ca^{2+} regulatory mechanisms is reported elsewhere in rat hearts following 12 weeks of treadmill training [34]. Thus, these findings may support the incorporation of RT into the treatment of T1DM, since slowed Ca^{2+} clearing and abnormal cardiomyocyte excitation-contraction coupling are prominent in T1DM [35].

The finding that rates of pressure development and relaxation were increased in D-RT and D-ART despite no improvement in glycemia (fructosamine) indicates that other factors may contribute to changes in rates of pressure development. For example, cardiomyocytes from insulin-resistant rats have demonstrated mechanical defects and impaired Ca^{2+} handling [36, 37]. In the present investigation, we report a negative correlation between the degree of insulin resistance and the rate of developed pressure. Indeed, the experimental groups that demonstrated the greatest insulin sensitivity, D-RT and D-ART, also displayed the quickest rates of pressure development and relaxation. In the insulin-resistant state, impaired SERCA activity is well documented to contribute to cardiomyocyte dysfunction [38], and RT itself has been shown to increase SERCA expression [33]. In the present study, SERCA2 expression was not changed as a result of RT or ART. This lack of change may not reflect changes in the activity levels of this enzyme, as impaired SERCA activity has been reported in insulin-resistant animals despite normal protein content [38]. Nonetheless, the implications of insulin resistance in the recovery from an I-R injury are significant and require further investigation, given the emerging evidence of "double diabetes," a separate classification of patients with T1DM that exhibit both insulin deficiency and resistance [39].

In seeking to explain the mechanistic means by which a specific exercise training regime may prove to be more beneficial for the functional recovery of the heart during an I-R injury, we examined cardiac Hsp70 protein expression in each of the groups [40, 41]. We observed an increase in left ventricular Hsp70 content in D-AT_{high} compared to both sedentary T1DM groups (D-CIT and D-IIT). Our laboratory, as well as others, has established the importance of exercise-induced Hsp70 expression in recovery from an I-R injury [40, 41]. This finding is in line with previous work from our laboratory that demonstrated both short- and long-term aerobic exercise can result in increased Hsp70 in the hearts of insulin-treated T1DM rats [4, 5]. Further, we showed that D-AT_{high} had higher Hsp70 expression than D-RT which supports an earlier finding by our laboratory [4]. While it is not clear why differences in the expression of Hsp70 exist between exercise modalities, it may be reflective of frequency, duration, and/or intensity of the exercise. We have previously shown that antioxidant enzymes are elevated in the myocardium following AT_{high}, but not following RT [4]. Increases in myocardial antioxidant defenses have been shown to be dependent on the duration and frequency of training [42], while exercise-induced elevations in Hsp70 are known to be intensity-dependent [43]. It is plausible that D-RT did not undergo the same quantity or intensity of exercise as was achieved in D-AT_{high}.

The largest barrier to exercise prescription for individuals with T1DM is exercise-induced hypoglycemia [6]. Thus, independent of which exercise provides the largest cardiovascular benefit, the risk of exercise-induced hypoglycemia must also be considered. Similar to past findings [4], D-AT_{high} resulted in a significant drop in blood glucose immediately following exercise, while D-RT did not. Interestingly, the integration of RT and AT_{high} (D-ART) did not alter the abrupt drop in blood glucose in response to AT_{high}. Recently, our group has demonstrated that both sedentary and aerobically trained T1DM rats using CIT demonstrate hepatic glycogen deficiencies [15], similar to what has been reported using clinical populations [16]. Despite increased glycemic control in IIT, there was no difference in hepatic glycogen content between D-IIT and D-CIT. Further, exercise-trained T1DM rats, regardless of training modality, demonstrated significantly lower liver glycogen content. It is expected that the amount of insulin in circulation in the treatment groups contributed to different hepatic glycogen levels. Both D-CIT and D-IIT had similar exogenous insulin concentrations, which would in turn regulate glycogen storage by increasing the activity of glycogen synthase [44].

Moreover, independent of exercise modality, trained T1DM rats displayed the smallest amounts of hepatic glycogen, concurrent with the lowest exogenous insulin requirements. Despite the apparent cardiovascular benefits associated with regular exercise, the decreased hepatic glycogen content in trained T1DM rats could have implications for combatting hypoglycemia, since hepatic glycogen is a prominent source of blood glucose during glucose-demanding states [17, 18]. However, it is important to note that T1DM rats in each of the different training modalities failed to reach hypoglycemic blood glucose concentrations (less than 3 mmol/L).

In conclusion, the first objective of the present investigation was to determine if AT_{high} coupled with CIT resulted in larger cardioprotective benefits than IIT alone. Findings presented here demonstrate that when CIT was paired with AT_{high}, the increase in cardioprotection from an I-R injury was similar to that of D-IIT. In fact, the current findings may suggest that CIT with AT_{high} may lead to a larger cardiac improvement in T1DM rats than IIT alone, given the potential role of elevated expression of left ventricular Hsp70 with this form of exercise. For the second objective, we determined that following long-term exercise training, both D-ART and D-RT resulted in similar levels of overall cardioprotection as D-AT_{high}; although each exercise training modality did appear to provide unique benefits. For example, improved glycemic control was only evident in D-AT_{high}, while the largest improvements in insulin sensitivity measures were evident in exercises that utilize resistance exercise (D-ART, D-RT). This study underlines the need to consider other factors besides glycemic control (i.e., insulin resistance) when tailoring an exercise treatment program for the patient with T1DM to reduce the risk of developing CVD.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This study was supported by Natural Sciences and Engineering Council Discovery Grant (RGPGP-2015-00059).

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