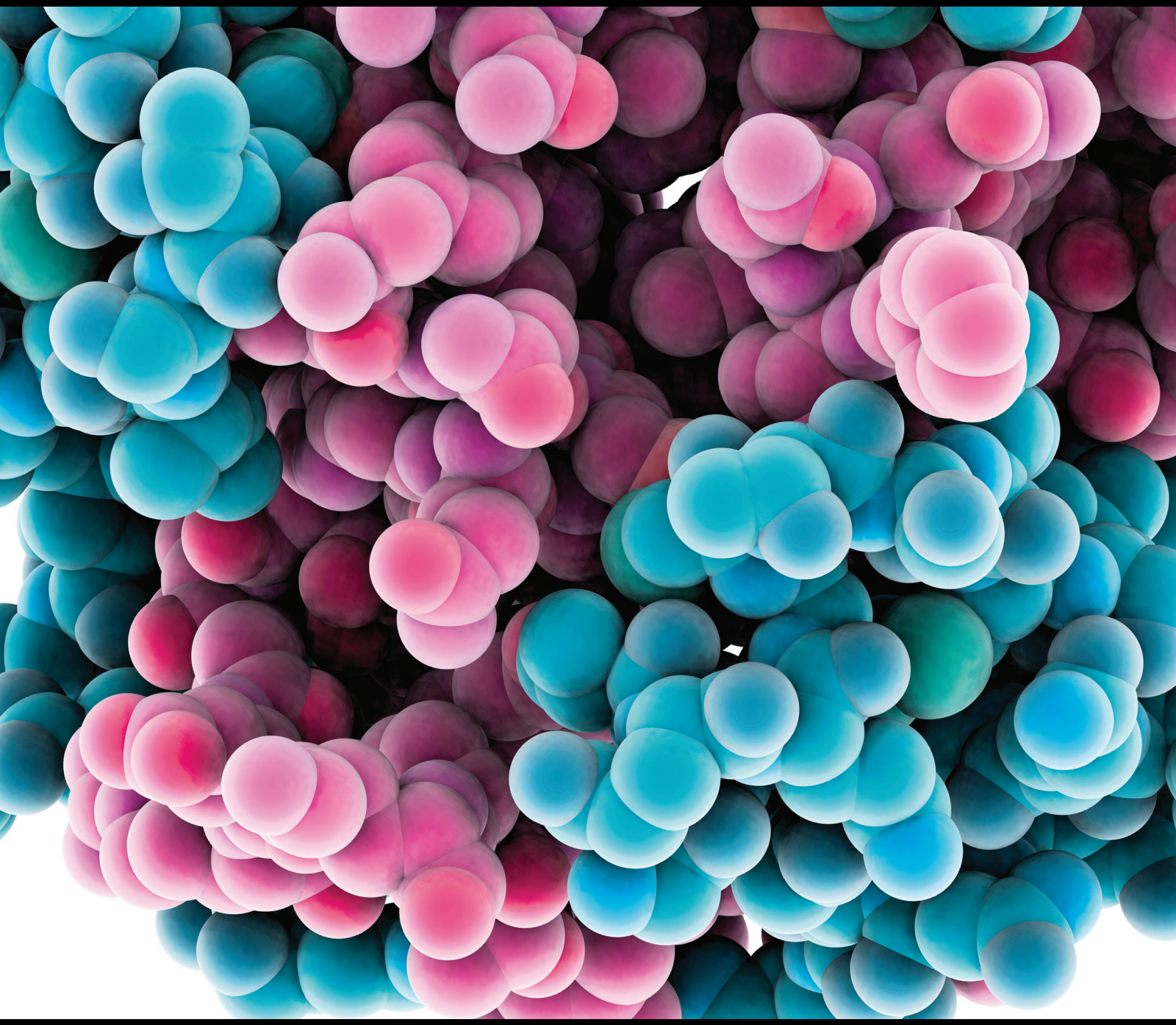
Journal of Diabetes Research

Interactions between Diabetes and the Heart

Guest Editors: John Skoularigis, Andreas Melidonis, Dirk Westermann, Vasiliki V. Georgiopoulou, Georgios Karagiannis, and Gregory Giamouzis



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Editorial Board

Contents

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Editorial

Interactions between Diabetes and the Heart

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Diabetes is a chronic metabolic disease that has reached epidemic proportions, affecting millions of individuals worldwide, and the global prevalence of the disease is expected to increase exponentially. Diabetes is associated with substantial mortality, morbidity, and healthcare expenditure.

Cardiovascular complications are the major cause of mortality and morbidity in individuals with diabetes and the largest contributor to the overall cost of diabetes. More than 60% of deaths in patients with diabetes have an underlying cardiovascular cause. Patients with diabetes have 2- to 4-fold higher risk of developing cardiovascular disease than individuals without diabetes [1]. Atherosclerosis has long been recognized as the main causative factor for cardiovascular events in patients with diabetes. Emerging evidence suggests that changes in circulating blood (e.g., inflammatory mediators, altered platelet function, hypercoagulability, hypofibrinolysis, and microparticles) and myocardial factors (e.g., altered metabolic state and neural and vascular impairment) may also play equally important roles in the pathophysiologic process underlying the increased propensity for cardiovascular disease in individuals with diabetes.

In this special issue, we have invited a few papers that address such issues.

Heart Failure and Diabetes. People with diabetes have an increased risk of developing heart failure, both as a consequence of coronary artery disease and as a result of cardiomyopathy, which may be due to a microangiopathy or primary cardiomyocyte dysfunction. Indeed, diastolic left ventricular dysfunction is a consistent observation through all levels of glucose intolerance [2]. Dr. K. Hensel et al. investigated whether speckle tracking echocardiography can be used to detect subclinical alterations of left ventricular myocardial deformation in asymptomatic pediatric patients with uncomplicated T1DM. Furthermore, they combined speckle tracking echocardiography with physical stress testing in order to unmask subtle changes of cardiac contractility that might potentially be occult at rest (for and against the existence of diabetic cardiomyopathy). With their results they provide further evidence for diabetes-associated nonischemic cardiomyopathy in T1DM patients as they describe the early stage of diabetic cardiomyopathy which is initiated by hyperglycemia, has insignificant changes in myocardial structure (normal left ventricular dimensions, wall thickness, and mass), has possible substructural changes in myocytes, and can only be detected by sensitive methods such as strain, strain rate, and myocardial tissue velocity. In another study Dr. L. León et al. discuss the current scientific evidences to propose circulating micro-RNA as promising biomarkers for early detection of diabetic cardiomyopathy and then to identify patients at high risk of diabetic cardiomyopathy development. Moreover, they summarize the research strategies to identify micro-RNA as potential biomarkers, the present limitations, challenges, and future perspectives.

Volatile anesthetics, like sevoflurane, have cardiodepressive effects and may aggravate cardiovascular complications

intraoperatively. Preservation of myocardial perfusion during surgery is particularly important in patients with increased risk for perioperative complications, such as diabetes. As sevoflurane's vasodilatory impact may be more abundant in patients with cardiometabolic disease, like T2DM, Dr. C. E. van den Brom et al. investigated the additional effect of sevoflurane anesthesia on myocardial perfusion and function in diet-induced prediabetic rats.

Hyperglycemia is associated with altered myocardial substrate use, a condition that has been hypothesized to contribute to impaired cardiac performance. It is caused to a great extent by change of energy metabolism, which is an additional trigger of functional and structural disorders of heart muscle. In turn, remodeling of the cardiomyocyte membranes with advanced glycation end products and free radical oxidation is essential factor in development of diabetes mellitus. Dr. S. Afanasiev et al. showed that in the experimental conditions induction of DM on the stage of formation of postinfarction remodeling increases adaptive ability of myocardium. It is manifested in inhibition of increase in lipid peroxidation processes activity and maintaining of force-interval reactions of myocardium connected with calcium transport systems of cardiomyocyte sarcoplasmic reticulum.

Antiplatelet Therapy in Diabetes. The ADP receptor P2Y12 plays a pivotal role in platelet aggregation [3]. This role is emphasized by the results of clinical trials that demonstrate improvement of long-term clinical outcomes in patients treated with the P2Y12 receptor antagonist clopidogrel. However, a high interindividual variability in platelet response to clopidogrel has been described. The fact that subjects with suboptimal platelet inhibition by clopidogrel are at increased risk of cardiovascular ischemic events represents an alarming clinical problem [4]. T2DM was recently connected with a failure in antiplatelet response to clopidogrel and the presence of high on-treatment platelet reactivity was repeatedly associated with the risk of ischemic adverse events. Patients with T2DM show significantly higher residual platelet reactivity on ADP receptor blocker therapy and are more frequently represented in the group of patients with high on-treatment platelet reactivity. The article by Dr. M. Samoš et al. reviews the current knowledge about possible interactions between T2DM and ADP receptor blockers therapy.

Heart Rate and Diabetes. Elevated resting heart rate has been associated with increased risk of all-cause mortality and cardiovascular events in healthy subjects as well as those with preexisting cardiovascular disease including hypertension, acute myocardial infarction, and heart failure or left ventricular dysfunction by numerous epidemiological studies [5]. However, limited data are available in T2DM patients. In an interesting study, Dr. V. Bartáková et al. evaluated whether resting heart rate could be a predictor of major cardiovascular events, progression of diabetic kidney disease, and all-cause mortality in a cohort of T2DM patients.

Adipose Tissue and Insulin Resistance. Adipose tissue accumulates both within and around internal organs with potential to negatively alter their biological function. Functional abnormalities of the adipose tissue are linked to inflammation, metabolic dysregulation, vascular dysfunction, impaired angiogenesis, and insulin resistance. Interest has focused on the potential role of epicardial adipose tissue as a modulator of cardiovascular function, owing to its immediate anatomic proximity to the coronary vasculature and myocardium with shared microcirculation [6]. Evidence suggests that epicardial adipose tissue could play a role in the pathogenesis of cardiovascular disease and is also potentially involved in the onset and progression of coronary artery disease. Increased expression of receptor for advanced glycation end products (RAGE) in adipose tissue has been associated with inflammation, adipocyte hypertrophy, and impaired insulin signal. The potential role of RAGE in epicardial adipose tissue has not been explored much. The study by Dr. E. Dozio et al. examined the RAGE expression in epicardial adipose tissue and suggests potential involvement in promoting epicardial adipose tissue dysfunction in coronary artery disease patients.

We hope that the readers of the journal will find the topics as interesting and important as we did.

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Research Article

Coupling of the Functional Stability of Rat Myocardium and Activity of Lipid Peroxidation in Combined Development of Postinfarction Remodeling and Diabetes Mellitus

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Coupling of the functional stability of rat myocardium and activity of lipid peroxidation processes in combined development of postinfarction remodeling and diabetes mellitus has been studied. The functional stability of myocardium was studied by means of the analysis of inotropic reaction on extrasystolic stimulus, the degree of left ventricular hypertrophy, and the size of scar zone. It was shown that in combined development of postinfarction cardiac remodeling of heart (PICR) with diabetes mellitus (DM) animal body weight decreased in less degree than in diabetic rats. Animals with combined pathology had no heart hypertrophy. The amplitude of extrasystolic contractions in rats with PICR combined with DM had no differences compared to the control group. In myocardium of rats with PICR combined with DM postextrasystolic potentiation was observed in contrast with the rats with PICR alone. The rats with combined pathology had the decreased value of TBA-active products. Thus, the results of study showed that induction of DM on the stage of the development of postinfarction remodeling increases adaptive ability of myocardium. It is manifested in inhibition of increase of LPO processes activity and maintaining of force-interval reactions of myocardium connected with calcium transport systems of sarcoplasmic reticulum of cardiomyocytes.

1. Introduction

Diabetes mellitus (DM) is one of the threatening factors which increases the risk of cardiovascular accidents during cardiovascular diseases [1]. Metabolic changes developing during diabetes mellitus aggravate disorders of functional state of cardiomyocytes in heart failure (HF) [2–4]. It is caused to a great extent by change of energy metabolism, which is an additional trigger of functional and structural disorders of heart muscle. In turn, remodeling of the cardiomyocyte membranes with advanced glycation end products and free radical oxidation is essential factor in development of diabetes mellitus [5, 6]. All these factors contribute to the disorder of electrical stability of membranes and the ionic balance of heart cells. These changes may define mainly cardiomyocyte contractility. The key structure, responding to intracellular transport of Ca^{2+} and, accordingly, to inotropic response of cardiomyocytes, is sarcoplasmic reticulum (SR) [7]. It has been shown that disorder of SR functions is accompanied by the inversion of force-frequency and force-interval dependences of myocardium [8, 9]. The interrelation between change of Ca^{2+} homeostasis in cardiomyocytes and progression of HF is revealed: disorder of intracellular Ca^{2+} transport precedes the depression of mechanical performance of heart [10–12].

An important role in disorder of ion transport systems of cardiomyocytes is played by lipid peroxidation (LPO) processes [13]. Intensification of LPO is nonspecific cell reaction to pathological actions. Development of HF and DM is accompanied with considerable increase of LPO activity [14, 15]. So, it is shown that LPO products act on lipid phase of membranes making it penetrable for hydrogen and calcium ions. It results in uncoupling of oxidative phosphorylation in mitochondria which leaves cell in the state of energy deficiency. At that state the excess amount of Ca^{2+} entering the cytoplasm is not able to be withdrawn from myoplasm and, subsequently, damages cellular structures.

In contrast to clinical data, which unambiguously points to the decrease of stability of diabetic heart to ischemia, results of experimental studies are sufficiently contradictory.

So, in number of researches one notes paradoxically high myocardial resistance to ischemia (in vivo and in vitro) in adult animals with short-term streptozotocin-induced diabetes [16–18]. Our preliminary study also revealed facts of the maintenance of the myocardial contractility in combined development of HF and DM. Mechanisms of this phenomenon remain subject for scientific research. States of Ca^{2+} transport systems of cardiomyocyte SR and activity of LPO processes in combined development of HF and DM have been studied insufficiently.

2. Materials and Methods

The study was performed on adult male Wistar rats 200–220 g. Four groups of animals were formed: first group consisted of intact rats ($n = 12$), second group of the rats with postinfarction cardiac remodeling (PICR) ($n = 11$), third group of the rats with induced DM ($n = 8$), and the IV group of the rats with DM induced 2 weeks after coronary occlusion ($n = 8$). By the time of the experiment all animals were of the same age. Myocardial infarction was induced by means of occlusion of the left anterior descending artery [19]; then the animals were housed under standard vivarium conditions. Diabetes mellitus was induced by single injection of 60 mg/kg dose of streptozotocin ("Sigma," USA) abdominally, diluted ex tempore with 0.01 M/L citrate buffer (pH 4.5). Rats of the IV group were taken in the experiment 6 weeks after induction of diabetes. Concentration of glucose in blood serum was defined by enzymatic-colorimetric test ("Biocon Diagnostic," Germany).

The development of heart and left ventricle hypertrophy was estimated by corresponding mass ratio [20]. For that reason the ratios of heart mass to animal body mass and left ventricle mass to heart mass were defined. Size of postinfarction scars of animal heart was estimated by the method of planimetry and calculated in percentages from area of free wall of left ventricle [21].

In the day of experiment animal blood has been sampled in a tube with heparin (10 : 1). Blood samples were centrifuged at 3000 rpm for 10 min. Obtained serum was dispensed for aliquots and stored in liquid nitrogen until the investigation moment.

Contractile activity was studied on papillary muscles. For that animals under Rausch-narcosis were immobilized with displacement of cervical region of the vertebral column and then their chests were opened. Isolated heart was washed in the specialized flow chamber through aorta with Krebs-Henseleit solution of the following composition (in mM): NaCl: 120; KCl: 4.8; $CaCl_2$: 2.0; $MgSO_4$: 1.2; KH_2PO_4: 1.2; $NaHCO_3$: 20.0; glucose: 10.0 ("Sigma," USA). Then, papillary muscles were isolated and placed in the temperature-stabilized (36°C) flow chamber. Perfusion of muscles has been performed with Krebs-Henseleit solution. Oxygenation of solution has been performed with carbogen (O_2: 95%, CO_2: 5%). Contractile activity of muscles was estimated in isometric mode, using "Force transducer KG-Series" transducer (Scientific Instruments GmbH, Germany). Tension developed by muscle calculated on diameter of isolated muscle (mN/mm^2) was estimated. Stimulation of muscles was performed with rectangular electrical pulses with duration of 5 ms and frequency of 0.5 Hz. Before the beginning of the research muscles had been adapted to the perfusion conditions and isometric mode in 60 minutes.

It is known that functional state of isolated myocardial strips can be estimated by changing the mode of their electrical stimulation. At extrasystolic impact, we registered extrasystolic contraction which characterizes excitability of sarcolemma [22] and postextrasystolic contraction which reflects the ability of cardiomyocyte sarcoplasmic reticulum (SR) to accumulate Ca^{2+} ions which additionally enter the myoplasm at extraordinary excitation and define amplitude of postextrasystolic contractions [22]. In our work, extrasystolic impact was made by additional single electrical pulse on 0.2, 0.225, 0.25, 0.5, 0.75, 1.0, and 1.5 s (extrasystolic interval) from the beginning of the regular cycle. Amplitudes of extrasystolic (ES) and postextrasystolic (PES) contraction were expressed as percentages of the amplitude of regular (basic) cycle. We analyzed the dependence of changes of ES and PES contraction amplitude on the duration of extrasystolic interval.

LPO activity in blood serum was estimated by measuring the concentration of TBA-active products (TBAAP) acquired in reaction with 2-thiobarbituric acid (TBA) [23]. The concentration of primary products of LPO-dien conjugates (DC) was measured in the hexane extracts of serum samples with spectrophotometer at 232 nm [24].

Data is presented in the form of median and interquartile range (Me (Q1; Q3)). Student's criterion has been used for normal distribution of values. Study data is presented as M ± SD, where M is mean value and SD is standard deviation. Reliability of differences of obtained data was estimated using Mann-Whitney U test for independent samples in the case of distribution which differs from normal one. Differences at value $p < 0.05$ have been considered statistically significant.

3. Results and Discussion

Results reflecting values of mass indices obtained in considered groups are presented in Table 1. It can be seen that the animals with PICR (the II group) had decreased (on 18.8%) body weight and hypertrophied (on 90%) heart compared to those of intact animals. Induction of diabetes (the III group) led to decreased animal body weight by 56%, $p < 0.05$, but in this case without heart hypertrophy. In combined development of PICR with DM (the IV group), animal body weight was 26% less than the one of the animals from the I group. These animals as well as the animals in the III groups did not have heart hypertrophy. It appeared that size of scar zone in II and IV groups did not differ. Blood glucose level of the animals of III and IV groups exceeded that of intact rats by 4.5 and 3 times, accordingly.

In our study, the remodeling of myocardium both after coronary artery occlusion (the II group) and after development of hyperglycemia (the III group) led to a change in inotropic reaction of papillary muscles on extrasystolic actions compared to the control group (Figure 1). So, amplitude of ES contractions of papillary muscles of the PICR

Table 1: Body and heart weights of rats after coronary artery occlusion and diabetes induction.

Number	Group	n	Body weight, g	Glucose, mol/L	Heart weight/body weight, mg/g	Left ventricle weight/heart weight, mg/mg	Scar area, %
I	Control	12	298 ± 23.7	6 ± 0.37	3.29 ± 0.21	0.645 ± 0.013	—
II	PICR	11	242 ± 11.17$^{*\#}$	7 ± 0.13$^{\#}$	6.27 ± 0.33$^{*\#}$	0.687 ± 0.016**	51.3 ± 8.9
III	DM	8	160 ± 14.8*	27 ± 2.75*	3.77 ± 0.31	0.676 ± 0.014	—
IV	PICR + DM	8	221 ± 4.51*	18 ± 1.79*	3.37 ± 0.11	0.673 ± 0.019	46.1 ± 2.7

Note. PICR: rats with postinfarction cardiac remodeling. $^{*}p < 0.01$, $^{**}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with PICR. Scar area was calculated as a percentage from area of free wall of left ventricle.

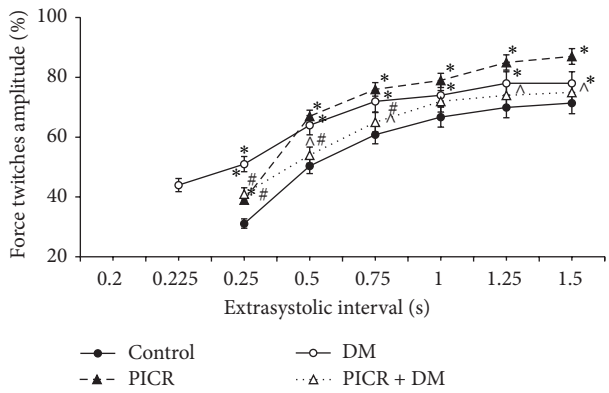

Figure 1: Extrasystolic contractions of papillary muscles of rats with postinfarction heart failure and diabetes mellitus. Note: the force twitches amplitude expressed in percentage of base contraction. $^{*}p < 0.001$ compared with control, $^{\#}p < 0.05$ compared with DM, and $^{\wedge}p < 0.05$ compared with PICR.

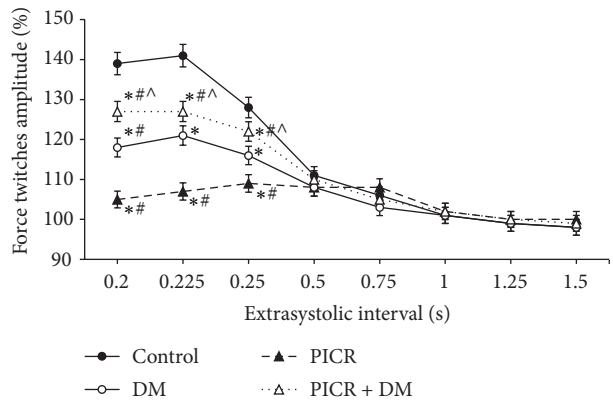

Figure 2: Postextrasystolic contractions of papillary muscles of rats with postinfarction heart failure and diabetes mellitus. Note: $^{*}p < 0.001$ compared with control, $^{\#}p < 0.05$ compared with DM, and $^{\wedge}p < 0.05$ compared with PICR.

rats (the II group) on short extrasystolic intervals was 8% higher than that of intact animals ($p < 0.05$). After the longest ES interval, this difference increased and reached 16% ($p < 0.05$). Amplitude increase of ES contractions of papillary muscles of PICR testifies the increased intracellular amount of Ca^{2+} taking part in ES contraction. It is known that ischemic damage of heart is characterized by the suppression of ATP-sensitive processes including the work of intracellular ion transport systems. It leads to the increase in intracellular concentrations of Na^{+} and Ca^{2+} [25–27]. ES contractions of papillary muscles of the rats from the III group have their own peculiarities. So, independent ES contraction appeared already at ES interval of 0.225 s. In the rest of the groups ES contraction appeared only at ES interval of 0.25 s. It is known that ES action causes inotropic response only if it happens in the phase of relative refractivity [22]. From these positions result obtained in the III group shows that development of diabetes leads to a shortened phase of absolute refractivity and hence to an increased excitability of cardiomyocytes. The fact that the amplitude of ES contractions in the III group on short extrasystolic intervals was 20% higher than that in the I group (intact animals) testifies in favor of that. At long intervals these differences decreased to 7% (Figure 1).

Result obtained by studying the IV group differs from the case of II or III group. In case of combined development of ischemic and diabetic damage of myocardium we obtained essentially less manifested change of ES contraction dynamics.

It is known that stimulating pulse which falls on the 3rd phase of action potential is not able to induce contractile response. However, it initiates additional income of external calcium ions in the myoplasm. This Ca^{2+} is accumulated in SR and takes part in the first PES cycle of contraction-relaxation [22]. For this reason amplitude of PES contraction exceeds amplitude of regular cycle. In our research extraordinary impetus at ES interval of 0.2 s did not cause ES contraction of the myocardium of intact rats (the I group). But we registered 39% increase of PES contraction amplitude compared to the amplitude of regular contraction (Figure 2). With appearance of ES contraction and increase of its amplitude we observed decrease of ES contraction amplitude. For intact animals PES potentiation of contraction was absent on the longest ES intervals (Figure 2).

As we can see from Figure 2 in the II group of rats PES potentiation of contraction of papillary muscles was not observed no matter what the duration of ES interval was. This fact can testify essential decrease of Ca^{2+} storing function of SR. Probably, in conditions of postinfarction remodeling of rat myocardium the function of Ca^{2+} transport systems of SR is damaged [11, 28, 29]. While studying the papillary muscles of the rats of the III group, PES potentiation of contraction was essentially lower than in the I group (intact animals) and was 21–16% (Figure 2). In combined postinfarction and

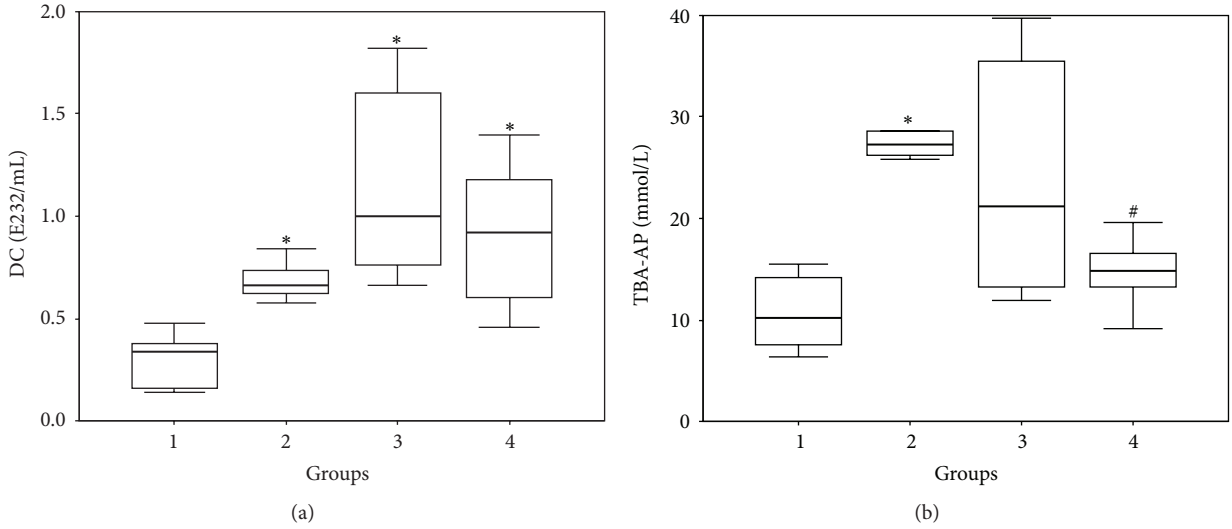

Figure 3: Concentration of DC (a) and TBA-AP (b) in blood plasma of the experimental animals (Me (Q1; Q3)). Note: (1) group: control, (2) group: PICR, (3) group: DM, and (4) groups: PICR + DM. $^{*}p < 0.01$ compared with group 1 (control) and $^{\#}p < 0.01$ compared with group 2 (PICR).

diabetic remodeling of myocardium (IV group) on the short ES intervals, the increase in PES contraction was 27–19% (Figure 2). This result testifies maintenance of Ca^{2+} storing ability of SR.

It is known that higher activity of peroxidation process is important component of damage of cardiomyocytes due to myocardium infarction [30]. Alteration of the lipid bilayer of membranes with oxygen radicals is considered to be one of the mechanisms of distortion of intracellular Ca^{2+} homeostasis and contractile activity of cardiomyocytes. Previously we have shown that higher activity of LPO is also maintained during postinfarction remodeling of heart. Moreover, in simulation of PICR, the dynamics of changes in LPO, the products (TBAAP and DC) in myocardial tissue, and blood serum of rats coincided [31]. On this basis it is possible to define TBAAP and DC concentration in blood serum and to extrapolate it on myocardium.

It is known that activation of free radical oxidation of lipids is also noted at DM [15]. Data obtained at determination of TBAAP and DC concentration in blood serum of animals included in the present research is presented in Figure 3. It can be seen that the PICR animals blood (the II group) contained reliably more LPO products than the intact animal group. Simulation of DM (the III group) also promoted reliable increase of TBAAP and DC concentration. Intensified generation of active oxygen forms and activation of LPO processes at the following pathologies is known fact and is noted in the works of many authors [13–15]. Active oxygen forms in pathologically high concentrations go into reaction and damage both lipids and proteins of cellular membranes and components of blood serum. Literature contains data about decreased activity of proteins and enzymes including Ca^{2+}-ATPase of cardiomyocytes [13] in pathologies accompanying activation of free radical processes. These results are quite matched with data obtained at estimation of inotropic reaction of papillary muscles of the animals of II and III groups on extrasystolic action. This reaction can be a consequence of decrease in activity of Ca^{2+}-ATPase and contractile proteins as the result of structural damage caused by active forms of oxygen, violation of lipid bilayer of membrane, and leakage of Ca^{2+} from sarcoplasmic reticulum.

Combining development of PICR and DM in theory should cause more manifested LPO activation. However, for animals with combined pathology (the IV group) we obtained paradoxical result. Thus, the value of TBAAP appeared reliably lower than in the II group. Also, the downward trend in DC concentration takes place. Obtained data is well-matched with the results characterizing contractile ability of papillary muscles of the animals of the IV group. Decreased TBAAP concentration testifies the decreased intensity of passing of concluding stages of lipid peroxidation reaction. The fact of negligible decrease in DC testifies that intensity of the first LPO stages remains on sufficiently high level. Metabolites of fat acids forming on these stages can take part in formation of other LPO products [32].

Our data testify that induction of diabetes on the background of postinfarction remodeling paradoxically promotes maintaining functional activity of Ca^{2+} transport systems of SR. It may be connected with the fact that glycosylation products increase rigidity of cardiomyocytes membranes on the background of developing hyperglycemia. Enhancement of adaptive reactions at combined development of postinfarction and diabetic damage of myocardium can be connected with peculiarities of intracellular energy metabolism at given pathological states. So, increase of glucose level during the first stages of development of postinfarction cardiosclerosis allows activating glycolysis processes in cardiomyocytes. It is known that positive effect of glucose on the heart functioning in the experimental myocardial ischemia is connected with increase of glycolytic production of ATP [33, 34]. In combination with inhibition of LPO activity, shift of energy

metabolism to glycolytic production of ATP can help to obtain higher functional activity of Ca^{2+} transport system of SR at combined pathology. Data obtained by us corresponds to the results of other researchers. So, it was shown that ATP which is formed in glycolysis process is the irreplaceable source of energy for Ca^{2+} transport system of SR [35]. Increase of ischemic resistivity of myocardium was described for animals with short term of streptozotocin stimulated diabetes in vivo and in vitro [16, 36].

Thus, results of present study showed that in the experimental conditions induction of DM on the stage of formation of postinfarction remodeling increases adaptive ability of myocardium. It is manifested in inhibition of increase in LPO processes activity and maintaining of force-interval reactions of myocardium connected with calcium transport systems of cardiomyocyte SR.

Conflict of Interests

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

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Research Article

Subclinical Alterations of Cardiac Mechanics Present Early in the Course of Pediatric Type 1 Diabetes Mellitus: A Prospective Blinded Speckle Tracking Stress Echocardiography Study

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Diabetic cardiomyopathy substantially accounts for mortality in diabetes mellitus. The pathophysiological mechanism underlying diabetes-associated nonischemic heart failure is poorly understood and clinical data on myocardial mechanics in early stages of diabetes are lacking. In this study we utilize speckle tracking echocardiography combined with physical stress testing in order to evaluate whether left ventricular (LV) myocardial performance is altered early in the course of uncomplicated type 1 diabetes mellitus (T1DM). 40 consecutive asymptomatic normotensive children and adolescents with T1DM (mean age 11.5 ± 3.1 years and mean disease duration 4.3 ± 3.5 years) and 44 age- and gender-matched healthy controls were assessed using conventional and quantitative echocardiography (strain and strain rate) during bicycle ergometer stress testing. Strikingly, T1DM patients had increased LV longitudinal ($p = 0.019$) and circumferential ($p = 0.016$) strain rate both at rest and during exercise ($p = 0.021$). This was more pronounced in T1DM patients with a longer disease duration ($p = 0.038$). T1DM patients with serum HbA_{1c} > 9% showed impaired longitudinal ($p = 0.008$) and circumferential strain ($p = 0.005$) and a reduced E/A-ratio ($p = 0.018$). In conclusion, asymptomatic T1DM patients have signs of hyperdynamic LV contractility early in the course of the disease. Moreover, poor glycemic control is associated with early subclinical LV systolic and diastolic impairment.

1. Introduction

Type 1 diabetes mellitus (T1DM) is ranging among the most common chronic disorders of childhood and adolescence [1] with increasing incidence worldwide [2, 3]. Cardiovascular disease is the most common cause of death in diabetic patients and currently one of the leading causes of death overall in the industrialized world [4]. While ischemic events range highest in the list of diabetic cardiovascular complications [5], diabetic patients also develop heart failure in the absence of arterial hypertension and myocardial ischemia [6–8]. Even though the existence of "diabetic cardiomyopathy" in humans is a current matter of ongoing scientific controversy [9, 10], there is growing evidence for the assumption that diabetes can lead to systolic and diastolic cardiac dysfunction without other obvious causes for cardiomyopathy, such as overt ischemia, coronary artery disease, arterial hypertension, or valvular heart disease [11–16]. While there is a variety of causes contributing to diabetes-associated heart failure including impaired calcium homeostasis, enhanced fatty acid metabolism, suppressed glucose oxidation, altered intracellular signaling, and pathologic remodeling, the underlying pathophysiology of diabetic cardiomyopathy is still not well understood [8]. Whether uncomplicated diabetes mellitus already affects myocardial function in asymptomatic children at an early stage of the disease currently remains elusive [17]. Hence, children and adolescents with uncomplicated diabetes may serve as an ideal model to study the effect of diabetic metabolic conditions in the absence of potentially confounding ischemic events.

Myocardial deformation is a complex three-dimensional process influenced by heterogeneously organized heart muscle fibers. Measurements of left ventricular (LV) function are important for the evaluation, management, and estimation of prognosis in patients with various forms of cardiovascular disease [18]. However, ejection fraction (EF), the current echocardiographic gold standard for the assessment of systolic function, bears considerable limitations as a prognostic parameter [19] and does not correlate well with quantitative measures of functional capacity [20]. It uses a simplistic approach based on visual assessment of inward motion and wall thickening that underestimates the true complexity of myocardial contraction and suffers from significant inter- and intrarater variability [21]. Thus, subtle alterations in myocardial wall motion remain occult. Speckle tracking echocardiography (STE) is a quantitative diagnostic method for the assessment of myocardial deformation [22]. STE derived measurements correlate well with functional capacity [23] and feature promising inter- and intraobserver reproducibility [24]. Moreover, STE has been shown to detect subclinical systolic LV impairment in asymptomatic patients with preserved EF and arterial hypertension [25] or heart failure [26], respectively.

The aim of this study was to investigate whether STE can be used to detect subclinical alterations of LV myocardial deformation in asymptomatic pediatric patients with uncomplicated T1DM. Furthermore, we combined STE with physical stress testing in order to unmask subtle changes of cardiac contractility that might potentially be occult at rest.

2. Methods

2.1. Study Population. For this prospective diagnostic study we enrolled 40 consecutive children and adolescents with T1DM aged 6 to 17 years (mean age 11.5 ± 3.1 years; 40% female) and 44 age- and sex-matched healthy controls (mean age 11.4 ± 2.9 years; 45% female). Mandatory inclusion criteria in the study group were the diagnosis of insulin-dependent T1DM and a good general health state. Exclusion criteria were other past or present medical conditions that may affect the cardiovascular system such as congenital heart disease, systemic inflammatory disease, for example, history of Kawasaki disease, proteinuria, the use of any type of systemically acting medication (other than insulin for the study group), developmental delay, body mass index > 30 kg/m^2, submaximal effort during exercise testing, short leg length, or pathologic EKG-changes at rest or during exercise. None of the included patients suffered from sings of end-organ damage such as evidence of renal failure or retinal changes. Healthy control subjects had an entirely negative medical history with regard to the cardiovascular as well as to any other organ system. A written informed consent was obtained from each participant as well as from their legal guardian prior to inclusion in the study. Subsequently, a thorough history and physical examination as well as both resting and exercise echocardiography and EKG were obtained. The sample size was achieved by including all patients from the hospital's diabetes clinic that were willing to participate in the study. A priori study design was established dividing the diabetes population into subgroups of patients with a disease duration of less than 4 years (n = 23, 57.5%) and more than 4 years (n = 17, 42.5%) as well as a three-column stratification according to glycemic control with serum HbA_{1c} < 7.5% (n = 10, 25%), HbA_{1c} 7.5–9% (n = 19, 47.5%), and HbA_{1c} > 9% (n = 11, 27.5%). The study was approved by the Witten/Herdecke University ethics committee and carried out in accordance with declaration of Helsinki's ethical principles for medical research involving human subjects. The study was registered to the Witten/Herdecke University Ethics and Clinical Trials Committee and assigned the trial number 113/2013.

2.2. Conventional and Doppler Echocardiography. All examinations were performed with the commercially available ultrasound device iE33 by Phillips Ultrasound Inc., USA, using a S5-1 Sector Array transducer (Sector 1–5 MHz). All images were digitally recorded and transferred to an offline workstation for analysis, using XCelera Version 3.1.1.422 by Phillips Ultrasound Inc., USA. According to echocardiography guidelines a complete standard 2D study, as well as a spectral and color flow Doppler examination, was carried out [27]. Image acquisition was performed in the parasternal long axis view, three short axis views, and the apical 4-, 3-, and 2-chamber views. M-mode images were taken at level of the aortic valve and the LV for subsequent measurement of aortic root diameter, left atrial diameter, interventricular septum, LV cavity, and LV posterior wall. Fractional shortening, LV mass, relative wall thickness, LV end-diastolic/end-systolic volume, EF, stroke volume, and cardiac output were calculated. Utilizing pw-Doppler and pw-TDI E/A-ratio, E/E′-ratio, mitral deceleration time, and isovolumetric relaxation time were measured for the assessment of LV diastolic function as previously described [28]. All measurements were evaluated using Z-scores [29]. During the entire examination a particular focus was set on the exclusion of any congenital heart disease as well as morphological or functional abnormalities.

2.3. Speckle Tracking Echocardiography. Myocardial deformation parameters (strain and strain rate) were measured acquiring standard 2D grayscale LV images. Circumferential strain (CS) was assessed in the standard parasternal short axis at the mitral valve plane (SAXB) and the papillary muscle plane (SAXM). Longitudinal strain (LS) was measured with standard apical 4-chamber (AP4), 3-chamber (AP3), and 2-chamber (AP2) apical views using conventional B-Mode imaging as previously described [22]. Five consecutive heart beats synchronized to a continuous EKG were recorded with frame rate set between 60 and 90 frames per second as recently suggested [30]. Caution was paid to minimize artifacts and to reduce noise for most accurate 2D strain estimation. All loops were digitally stored anonymized in the DICOM format and transferred to an offline workstation for postprocessing using the commercially available software Qlab 9. Segmental and global LS and CS were measured in 7 and 6 segments per view, respectively, by manual tracing of the endocardial contour at end-systole. The following frames were automatically analyzed by temporal tracking of acoustic speckles that are individual to each segment of the myocardial

tissue. Real-time verification of adequate tracking and full thickness coverage of the myocardium including the epicardial and endocardial borders were optimized by manual readjustment of poorly tracked segments where necessary. More negative strain and strain rate values will be described as "higher" in this paper, even though mathematically it is vice versa, as more negative values represent an increased contraction of the myocardium.

Both resting and exercise echocardiographic images were additionally analyzed by a second, independent reader who was blinded to the results of the first examiner and the study group status of the respective echocardiographic image in order to determine interobserver reliability.

2.4. Quantitative Stress Echocardiography. After the general echocardiographic studies, participants pedaled in a supine position utilizing a standard cycle ergometer at approximately 60 rounds per minute against a ramp protocol with increasing resistance. Image acquisition for speckle tracking deformation analyses was carried out in the resting state and at the maximum level of physical exhaustion (≈2 Watt per kilogram body weight). A standardized pattern of consecutive images was acquired at each time point in the following order: SAXB, SAXM, AP4, AP2, and AP3. A 12-channel EKG was continuously monitored and blood pressure measurements were collected at 2-minute intervals.

All echocardiographic analyses were performed by the same investigators, who were blinded to the study group status at the time of the assessment of strain and strain rate. The results were reproducible and interobserver variability was below 5.8% in our study.

In order to reduce the risk of exercise induced hypoglycemia in diabetic patients, serum glucose levels should exceed 100 mg/dL to 150 mg/dL. Patients with blood sugar levels below 100 mg/dL were provided with extra carbohydrate exchange such as candy bars or orange juice prior to physical exercise testing.

2.5. Biostatistical Analysis. Baseline demographics, clinical data, hemodynamic parameters, and echocardiographic characteristics of the two groups were described by mean and standard deviation. Clinical parameters, hemodynamic data, and echocardiographic characteristics of the two study groups were compared using the Mann-Whitney U test. Wilcoxon signed-rank test was used for the measurement of the effect of exercise within one group. p values <0.05 constituted statistical significance. Box-Whisker-Plots were used for the graphic representation of the data distribution. SPSS Statistics for Macintosh, Version 22.0. (IBM Corporation, USA), was used for all statistical analyses.

3. Results

3.1. Epidemiological Data. Baseline demographic and hemodynamic data of the study population are summarized in Table 1. There was no significant difference in age, body weight, height, bmi, or the level of exercise routine between the two groups. Blood pressure and heart rate did not differ between the two groups at rest or during stress testing except for a slightly higher heart rate in T1DM patients at rest (84.4 ± 11.3 bpm) when compared to healthy controls (76.2 ± 9.3 bpm; $p = 0.001$). However, all baseline and hemodynamic parameters in both groups were within normal limits [31]. Mean disease duration in the study group was 4 ± 3.5 years and mean glycated hemoglobin (HbA_{1c}) was $8.3 \pm 1.2\%$. One patient was excluded from the study due to detection of a previously unknown valvular aortic stenosis.

3.2. Conventional Echocardiography. Conventional echocardiographic characteristics are outlined in Table 2. There are no significant differences of atrial/aortic diameters or LV function parameters such as fractional shortening, EF, stroke volume, and cardiac output, except for a marginally larger systolic diameter of the interventricular septum in the control group (1.17 ± 0.20 cm) when compared to T1DM patients (1.06 ± 0.22 cm). Yet all values were within the normal range evaluated by Z-scores [29]. Analysis of diastolic function showed a significantly decreased E/A-ratio in the T1DM group (1.6 ± 0.28) when compared to healthy controls (1.72 ± 0.26; $p = 0.031$). E/E′-ratio, IVRT, and mitral deceleration time as well as all other assessed parameters of diastolic function showed no significant differences between the two groups.

3.3. Speckle Tracking Stress Echocardiography. Myocardial deformation was quantitatively measured using speckle tracking echocardiography at rest and during physical stress testing on a bicycle ergometer. Results for peak LV myocardial *strain rate* are displayed in Table 3. T1DM patients were shown to have increased myocardial contractility both at rest ($p = 0.016$) and during stress ($p = 0.021$). While statistical significance was reached in 4 out of 14 comparisons, T1DM patients had higher circumferential and longitudinal *strain rate* than healthy controls in 13 out of 14 comparisons. The significance of the difference of AP2 derived longitudinal strain rate in the diabetic and control group at rest (-1.94 ± 1.14 versus $-1.54 \pm 0.25\,s^{-1}$) is limited by the nonnormal distribution of the parameters.

The effect of disease duration on LV myocardial contractility is demonstrated in Figure 1. While T1DM patients had overall higher global LV *strain rates* at rest when compared to healthy controls, patients with a disease duration of >4 years had significantly increased LV *strain rate* at rest ($p = 0.038$) and during exercise ($p = 0.05$). Figure 2 illustrates generic speckle tracking echocardiography images of increased peak LV systolic *strain rate* in a patient with T1DM and a healthy sex- and age-matched control subject.

Overall, peak LV myocardial *strain* was not shown to be statistically different between the two groups, neither at rest, nor during physical exercise (see Table 4). However, significant differences could be demonstrated when analyzing the T1DM group stratified by glycemic control as visualized in Figure 3. T1DM patients with serum $HbA_{1c} > 9\%$ had significantly depressed peak LV CS ($p = 0.005$) and LS ($p = 0.008$) when compared to diabetic patients with better glycemic control and healthy controls, respectively.

T1DM patients showed beginning impairment of LV diastolic function (E/A-ratio) when compared to healthy

Table 1: Baseline clinical characteristics and hemodynamics of the study population.

	Diabetes ($n = 40$)	Control ($n = 44$)	p value
Age (years)	11.5 ± 3.1	11.4 ± 2.9	n.s.
Height (cm)	153.0 ± 18.2	154.1 ± 16.8	n.s.
Weight (kg)	46.9 ± 16.9	48.0 ± 16.3	n.s.
Body surface (m^2)	1.4 ± 0.3	1.4 ± 0.3	n.s.
Body mass index (kg/m^2)	19.3 ± 3.1	19.6 ± 3.5	n.s.
Exercise routine (1: in school; 2: <3 times/week; 3: ≥3 times/week)	1.7 ± 0.8	2.0 ± 0.7	n.s.
Duration of disease (years)	4 ± 3.5	—	—
HbA_{1c} (%)	8.3 ± 1.2	—	—
Rest			
Heart rate (beats/minute)	84.4 ± 11.3	76.2 ± 9.4	0.001
BP systolic (mmHg)	105.7 ± 9.6	105.8 ± 9.2	n.s.
BP diastolic (mmHg)	58.5 ± 7.9	59.4 ± 9.2	n.s.
Low stress level			
Heart rate (beats/minute)	112.0 ± 9.3	108.6 ± 12.8	n.s.
BP systolic (mmHg)	122.3 ± 18.2	120.2 ± 16.9	n.s.
BP diastolic (mmHg)	65.2 ± 13.6	63.7 ± 11.0	n.s.
Level of resistance (W/kg body weight)	0.4 ± 0.3	0.5 ± 0.3	n.s.
High stress level			
Heart rate (beats/minute)	161.5 ± 13.1	156.8 ± 17.5	n.s.
BP systolic (mmHg)	148.1 ± 21.9	140.1 ± 22.9	n.s.
BP diastolic (mmHg)	74.9 ± 12.6	71.2 ± 13.8	n.s.
Level of resistance (W/kg body weight)	1.7 ± 0.4	1.8 ± 0.4	n.s.

p values calculated with the Man-Whitney U test, level of significance = 0.05.

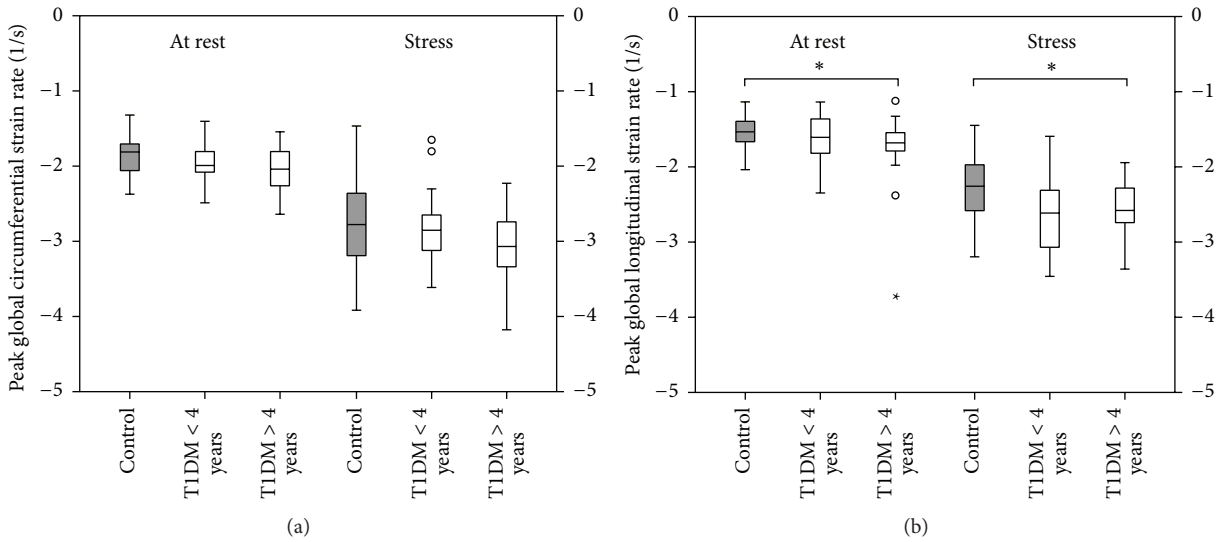


Figure 1: Peak systolic global left ventricular *strain rate* in relation to the duration of disease. Type 1 diabetic children ($n = 40$) have increased strain rate both at rest and during exercise when compared to healthy controls ($n = 44$). Patients with a disease duration >4 years ($n = 17$, 42.5%) exhibit higher strain rates than those with a disease duration <4 years ($n = 23$, 57.5%). (a) Peak systolic global LV circumferential *strain rate*. (b) Peak systolic global LV longitudinal *strain rate*. $^*p < 0.05$; p values were calculated with Mann-Whitney U and Wilcoxon signed-rank tests.

Table 2: Conventional echocardiographic parameters derived from two-dimensional and Doppler imaging.

	Diabetes ($n = 40$)	Control ($n = 44$)	p value
Aortic root (AoR) diameter (cm)	2.33 ± 0.35	2.41 ± 0.35	n.s.
Left atrial (LA) diameter (cm)	2.56 ± 0.38	2.71 ± 0.45	n.s.
LA/AoR	1.11 ± 0.16	1.13 ± 0.16	n.s.
Fractional shortening (%)	33.41 ± 4.08	34.78 ± 3.94	n.s.
Interventricular septal end-systolic diameter (cm)	1.06 ± 0.22	1.17 ± 0.20	0.011
Interventricular septal end-diastolic diameter (cm)	0.84 ± 0.18	0.89 ± 0.16	n.s.
LV end-systolic diameter (cm)	2.70 ± 0.43	2.76 ± 0.41	n.s.
LV end-diastolic diameter (cm)	4.05 ± 0.56	4.27 ± 0.46	n.s.
LV posterior wall diameter, systolic (cm)	1.23 ± 0.20	1.27 ± 0.21	n.s.
LV posterior wall diameter, diastolic (cm)	0.79 ± 0.16	0.81 ± 0.15	n.s.
LV mass (g)	102.74 ± 41.82	115.18 ± 37.56	n.s.
Relative wall thickness	0.20 ± 0.04	0.19 ± 0.03	n.s.
End-diastolic volume of the left ventricle (mL)	70.18 ± 24.66	79.63 ± 27.97	n.s.
End-systolic volume of the left ventricle (mL)	27.69 ± 9.82	31.66 ± 11.78	n.s.
Ejection fraction (%)	61.29 ± 4.77	60.16 ± 4.67	n.s.
Stroke volume (mL)	44.7 ± 14.4	49.3 ± 18.1	n.s.
Cardiac output (L/min)	3.7 ± 1.1	3.7 ± 1.3	n.s.
Mitral inflow: E-wave (cm/s)	95.36 ± 13.45	96.86 ± 14.26	n.s.
Mitral inflow: A-wave (cm/s)	60.84 ± 12.27	57.36 ± 10.41	n.s.
E-wave/A-wave	1.60 ± 0.28	1.72 ± 0.26	0.031
Mitral deceleration time (s)	0.17 ± 0.04	0.18 ± 0.04	n.s.
Isovolumetric relaxation time (s)	0.05 ± 0.01	0.05 ± 0.01	n.s.
S′ (cm/s)	7.92 ± 0.97	8.17 ± 1.19	n.s.
E′ (cm/s)	12.54 ± 1.81	13.03 ± 1.87	n.s.
A′ (cm/s)	5.42 ± 1.20	5.51 ± 1.11	n.s.
E′/A′ (cm/s)	2.44 ± 0.75	2.48 ± 0.72	n.s.
E/E′ (cm/s)	7.71 ± 1.20	7.56 ± 1.42	n.s.

p values calculated with the Man-Whitney U test, level of significance = 0.05.

controls (see Table 2). This effect was statistically significant for the comparison of the entire T1DM group to healthy controls ($p = 0.031$) and more pronounced in patients with poor glycemic control represented by serum HbA_{1c} levels > 9% ($p = 0.018$) as visualized in Figure 4.

4. Discussion

4.1. Type 1 Diabetic Children Have Increased Peak Left Ventricular Strain Rate. In order to assess the effect of type 1 diabetes mellitus on LV myocardial contractility in the absence of ischemic events early in the course of the disease we performed speckle tracking echocardiography in combination with ergometer stress testing in asymptomatic normotensive pediatric patients with uncomplicated T1DM and healthy controls. Interestingly and somewhat counterintuitively, we found diabetic children to exhibit LV systolic hypercontractility represented by overall increased peak circumferential and longitudinal *strain rate* both at rest and during exercise (see Table 3 and Figures 1 and 2). The observed statistically significant increases in LV strain rate in the T1DM group such as increased global longitudinal strain rate during stress testing (-2.59 ± 0.47 versus $-2.32 \pm 0.41\ s^{-1}$) should not be overinterpreted as a single finding with direct clinical implication but rather regarded as the tip of the iceberg of the overall tendency for T1DM patients to exhibit increased peak systolic LV strain rate. At first, this may seem surprising given the fact that diabetic cardiomyopathy potentially results in a gradual decline of myocardial function with the ultimate end-point of diabetic heart failure. However, we hypothesize that diabetic cardiomyopathy may in fact feature an early subclinical phase of paradoxical LV hyperdynamics as a sign of

Table 3: Speckle tracking derived peak systolic LV *strain rate* at rest and during stress testing.

	Diabetes ($n = 40$)	Control ($n = 44$)	p value
Rest			
Global circumferential strain rate (s^{-1})	−1.99 ± 0.28	−1.87 ± 0.24	n.s.
Circumferential strain rate (SAXM) (s^{-1})	−2.05 ± 0.35	−1.86 ± 0.25	0.016
Circumferential strain rate (SAXB) (s^{-1})	−1.96 ± 0.29	1.90 ± 0.31	n.s.
Global longitudinal strain rate (s^{-1})	−1.70 ± 0.44	−1.55 ± 0.21	n.s.
Longitudinal strain rate (AP4) (s^{-1})	−1.58 ± 0.34	−1.52 ± 0.28	n.s.
Longitudinal strain rate (AP2) (s^{-1})	−1.94 ± 1.14	−1.54 ± 0.25	0.019
Longitudinal strain rate (AP3) (s^{-1})	−1.64 ± 0.35	−1.63 ± 0.30	n.s.
Stress			
Global circumferential strain rate (s^{-1})	−2.92 ± 0.54	−2.76 ± 0.60	n.s.
Circumferential strain rate (SAXM) (s^{-1})	−2.92 ± 0.58	−2.73 ± 0.61	n.s.
Circumferential strain rate (SAXB) (s^{-1})	−2.86 ± 0.52	−2.66 ± 0.68	n.s.
Global longitudinal strain rate (s^{-1})	−2.59 ± 0.47	−2.32 ± 0.41	0.021
Longitudinal strain rate (AP4) (s^{-1})	−2.64 ± 0.53	−2.23 ± 0.37	0.002
Longitudinal strain rate (AP2) (s^{-1})	−2.40 ± 0.47	−2.47 ± 0.45	n.s.
Longitudinal strain rate (AP3) (s^{-1})	−2.71 ± 0.72	−2.60 ± 0.75	n.s.

SAXM: parasternal short axis view at the papillary muscle plane, SAXB: parasternal short axis view at the mitral valve plane, AP4: apical four-chamber view, AP2: apical two-chamber view, and AP3: apical three-chamber view; p values calculated with Man-Whitney U test, level of significance = 0.05.

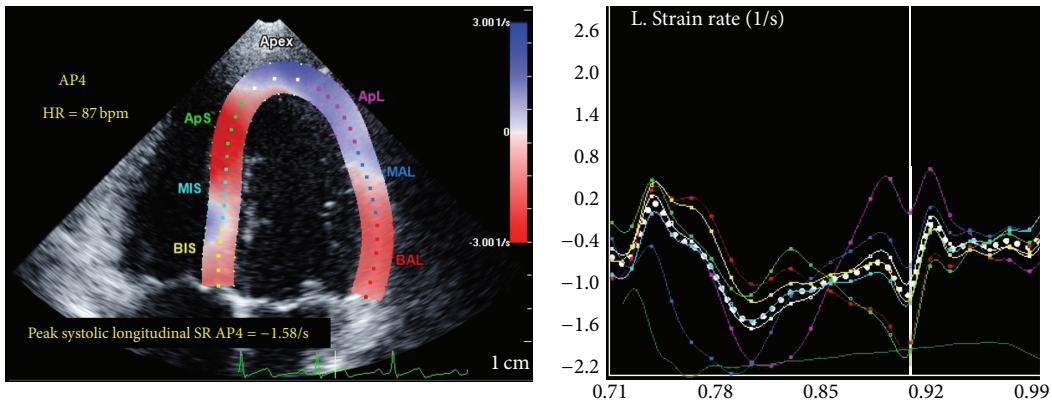
Table 4: Speckle tracking derived peak systolic LV *strain* at rest and during stress echocardiography.

	Diabetes ($n = 40$)	Control ($n = 44$)	p value
Rest			
Global circumferential strain rate (%)	−25.5 ± 3.3	−25.0 ± 3.4	n.s.
Circumferential strain rate (SAXM) (%)	−26.6 ± 4.7	−25.9 ± 3.9	n.s.
Circumferential strain rate (SAXB) (%)	−24.4 ± 3.2	−24.0 ± 4.4	n.s.
Global longitudinal strain rate (%)	−20.1 ± 2.3	−20.7 ± 2.5	n.s.
Longitudinal strain rate (AP4) (%)	−19.9 ± 2.5	−20.2 ± 3.0	n.s.
Longitudinal strain rate (AP2) (%)	−20.6 ± 3.2	−20.9 ± 3.1	n.s.
Longitudinal strain rate (AP3) (%)	−20.3 ± 2.3	−21.6 ± 2.8	n.s.
Stress			
Global circumferential strain rate (%)	−24.2 ± 3.9	−23.8 ± 4.1	n.s.
Circumferential strain rate (SAXM) (%)	−24.6 ± 4.0	−23.9 ± 4.6	n.s.
Circumferential strain rate (SAXB) (%)	−23.3 ± 4.1	−23.3 ± 4.3	n.s.
Global longitudinal strain rate (%)	−21.6 ± 2.9	−21.0 ± 2.7	n.s.
Longitudinal strain rate (AP4) (%)	−21.6 ± 3.1	−21.0 ± 2.3	n.s.
Longitudinal strain rate (AP2) (%)	−21.8 ± 3.7	−21.2 ± 0.5	n.s.
Longitudinal strain rate (AP3) (%)	−21.7 ± 3.7	−21.7 ± 4.4	n.s.

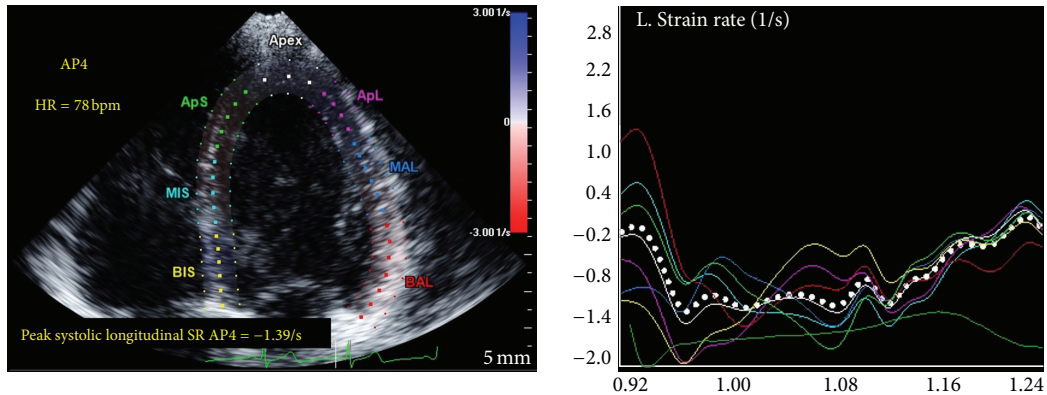
SAXM: parasternal short axis view at the papillary muscle plane, SAXB: parasternal short axis view at the mitral valve plane, AP4: apical four-chamber view, AP2: apical two-chamber view, and AP3: apical three-chamber view; p values calculated with Man-Whitney U test, level of significance = 0.05.

impaired mechanical efficiency long before long-term deterioration of myocardial function becomes evident. While most studies focus on intermediate or late stage disease reporting of depressed LV systolic function, there are a number of human and animal model studies in favor of our hypothesis.

Chung et al. found increased LV torsion despite preserved EF, circumferential strain, and longitudinal shortening using tagged MRI in young adult patients with tightly controlled T1DM [32]. Similarly, a stress MRI spectroscopy study revealed a reduced phosphocreatine/γ-ATP ratio as a sign of altered myocardial energetics in young adults with uncomplicated T1DM, independent of coronary microvascular function [33]. In another tagged MRI study hyperdynamic LV twist mechanics were described in coexistence with



(a) Type 1 diabetes mellitus



(b) Control

Figure 2: Speckle tracking echocardiography at rest in the apical 4-chamber view. (a) Peak systolic global LV longitudinal *strain rate* in a pediatric patient with type 1 diabetes mellitus. (b) Peak systolic global LV longitudinal *strain rate* in a healthy control subject. Dotted white line: global longitudinal *strain rate*, the coloured lines on the right correspond to the myocardial segments indicated on the left, dark green line at the bottom: ECG. Note the increased peak early systolic strain rate in the diabetic patient.

signs of altered myocardial perfusion in young patients with uncomplicated T1DM [34]. Moreover, our results are in accordance with two conventional echocardiographic studies demonstrating increased LV contractility in diabetic children without arterial hypertension, ischemic heart disease, or nephropathy using M-mode and Doppler imaging [35, 36]. Furthermore, our finding of LV hyperdynamic contractility early in the course of diabetes mellitus is in agreement with results from animal model studies in leptin receptor-deficient mice utilizing in vivo catheterization. Buchanan et al. discovered diabetes-associated LV hypercontractility as an indication for altered myocardial substrate use and reduced myocardial efficiency in hyperglycemia. The phenomenon occurred early and slightly faded subsequently [37]. Additionally, Van den Bergh et al. described impaired mechanical efficiency and increased ventriculoarterial coupling that was associated with altered cardiac loading conditions [38]. Therefore, for the assessment of myocardial contractility in human subjects, a noninvasive measure that is least dependent on variations in LV loading must be utilized in order to minimize potential confounding.

Strain and strain rate are quantitative measures for the echocardiographic assessment of myocardial deformation [39]. Strain is an index of deformation describing a percentage change from the original dimension. Strain rate is a measure of the rate at which this change happens and is expressed as per second (s^{-1}). While most studies assessing myocardial deformation in diabetic patients mainly focus on strain, strain rate is in fact a more robust index of LV myocardial contractility as it is less dependent on confounding factors such as pre- and afterload [40–43] and it is even more closely related to contractility than the widely used EF [44]. The present study is the first clinical study demonstrating overall increased strain rate in the early stage of human T1DM.

In contrast, Di Cori and colleagues used tissue-Doppler imaging to analyze myocardial deformation in adult T1DM patients (mean age 30 ± 4.1 years and mean disease duration 8.9 ± 3.7 years) and found depressed LV myocardial strain and equivocal strain rate [45]. There are several explanations for this deviation in deformation parameters. First, Di Cori and colleagues only assessed regional segmental strain (rate) of the midposterior septum (decreased in T1DM) and midlateral wall (increased in T1DM) and not global strain (rate). Given the strong heterogeneity of myocardial fiber organization in the LV, myocardial deformation naturally exhibits regional variations [46–48]. Thus, the assessment of

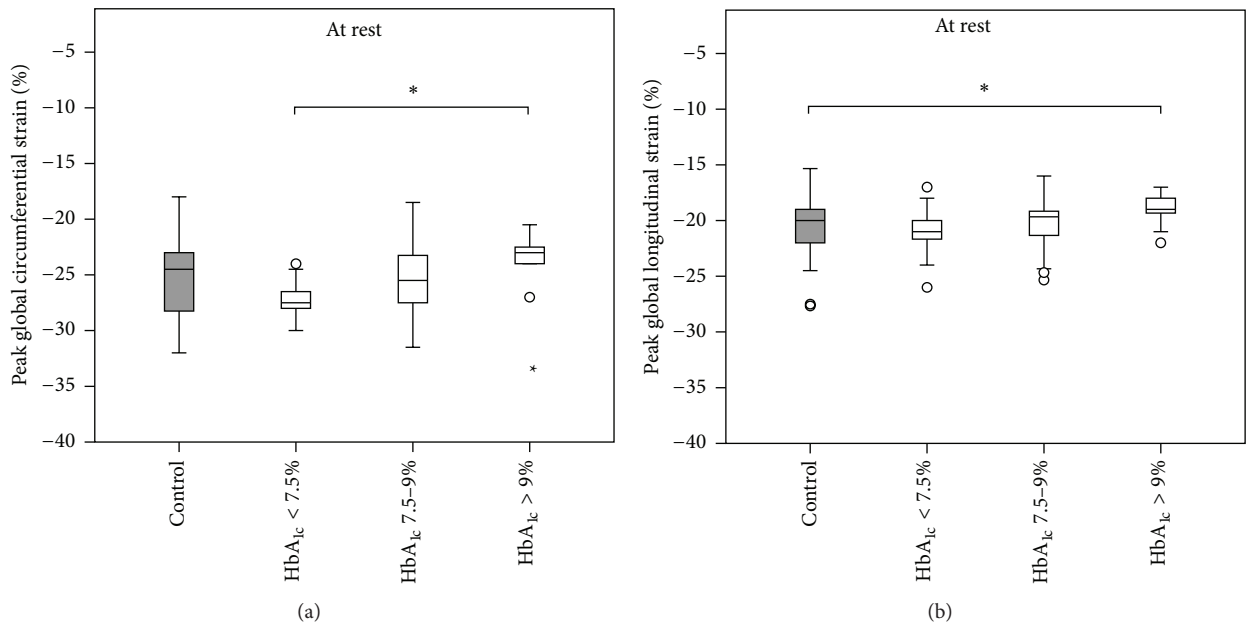


Figure 3: Peak systolic global left ventricular *strain* in relation to glycemic control. Type 1 diabetic children with poor glycemic control have decreased peak systolic global left ventricular *strain* when compared to healthy controls. (a) Peak systolic global LV circumferential *strain*. (b) Peak systolic global LV longitudinal *strain*. HbA_{1c} < 7.5% (n = 10, 25%), HbA_{1c} 7.5–9% (n = 19, 47.5%), and HbA_{1c} > 9% (n = 11, 27.5%); $^{*}p < 0.05$; p values were calculated with Mann-Whitney U and Wilcoxon signed-rank tests.

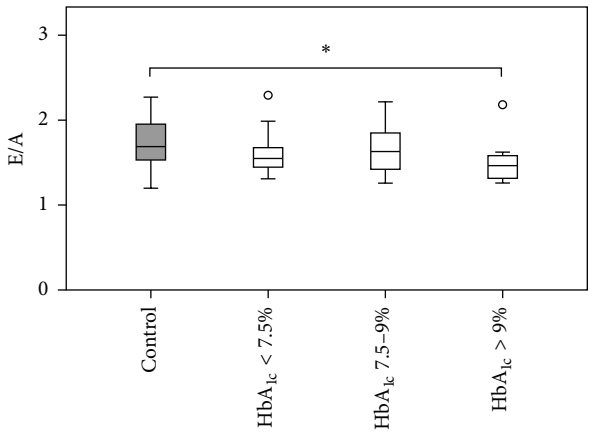


Figure 4: Left ventricular diastolic function (E/A-ratio) in relation to glycemic control. Type 1 diabetic children with poor glycemic control have echocardiographic evidence of impaired diastolic filling of the left ventricle in comparison to healthy control subjects. $^{*}p < 0.05$; p values were calculated with Mann-Whitney U and Wilcoxon signed-rank tests.

only two isolated segments likely is an oversimplification of the complex mechanism underlying LV myocardial contractility. Second, tissue-Doppler echocardiography was used, a method that bears considerable limitations such as angle-dependency and interrater variability [49]. Third, Di Cori and colleagues included an older diabetic study population with a markedly longer disease duration in comparison to the present study population. In our study the mean disease duration in the T1DM group was 4 ± 3.5 years. Interestingly, a subgroup analysis revealed that the increase in global LV peak longitudinal strain rate is statistically significant for T1DM patients with a disease duration >4 years (see Figure 1). Hence, it is well imaginable that the here described diabetes-associated cardiac changes require a certain time interval of a few years to become evident. Furthermore, the observed hypercontractility in T1DM in the present study is possibly a transient effect in the early phase of diabetic nonischemic cardiomyopathy that fades in the subsequent course of the disease, as observed by Di Cori and colleagues. Longitudinal studies are needed in order to further elucidate the natural course of diabetic cardiomyopathy throughout childhood and adulthood.

4.2. Left Ventricular Longitudinal and Circumferential Strain Is Impaired in Children with Poorly Controlled Type 1 Diabetes Mellitus. In this study there was no significant difference in overall peak LV longitudinal or circumferential strain between T1DM patients and healthy controls neither at rest nor during stress testing. This is in accordance with a Korean study of a very similar (age, disease duration, and glycemic control) T1DM population that also failed to demonstrate overall impairment of systolic strain [50]. Strain rate however was not measured in that study. While there is a considerable number of clinical studies reporting an impairment of (mainly global longitudinal) strain in diabetes mellitus type 1 [51–53] and type 2 [54], all of these studies either include adult patients and/or are confounded by longer disease duration [51, 55–57], LV structural abnormalities [51, 55, 58], impaired EF [52], obesity, arterial hypertension [51, 54, 59–61], nephropathy [51, 57, 61], heart failure [55], overt peripheral vascular disease [56], use of negatively

inotropic medications [51, 54, 60], or tobacco use [51]. Furthermore, in contrast to our study design all of the abovementioned studies are considerably limited by the fact that the echocardiographic interpreter was not blinded. This is a substantial limitation because speckle tracking derived myocardial deformation parameters are extremely sensible to manual adjustments.

Recently, a blinded speckle tracking study in 1065 normotensive T1DM patients (mean age 49.5 ± 14.5 years and mean disease duration 26.1 ± 15.7 years) convincingly demonstrated that the impairment of myocardial strain in T1DM is solely driven by the presence of albuminuria [62]. There was no difference in myocardial strain between T1DM patients without albuminuria and healthy controls. Strain rate however was not assessed in that study. As our study participants were screened negative for albuminuria and disease duration was considerably shorter than in the abovementioned study, the absence of overall impaired systolic strain in the present study population is not surprising. Moreover, our findings are in concordance with two MRI studies demonstrating preserved LV strain mechanics in young adult diabetic patients [63, 64].

Subdividing our T1DM population according to the degree of glycemic control, an association of both longitudinal and circumferential strains with serum levels of HbA_{1c} became evident (see Figure 3). This is in agreement with recent 3D speckle tracking studies demonstrating a negative impact of HbA_{1c} on LV myocardial strain in adult patients with diabetes mellitus [55, 65, 66]. Furthermore, this is underlined by prospective observational studies reporting the association of poor glycemic control with the development of heart failure in large cohorts of T1DM patients [5, 67]. In addition, several animal model studies are in accordance with our observations of diabetes-associated alterations in LV myocardial contractility [68, 69]. Accountable pathologic mechanisms are diabetes-induced loss of t-tubule structure [14], formation of advanced glycosylation end products with subsequent pathologically increased collagen cross-linking [70], altered mitochondrial energetics [71], and several other metabolic imbalances [11, 72]. The finding of overall preserved myocardial strain in the entire diabetic study population and decreased strain only in those subjects with poor glycemic control can be explained by the timing of the investigation. At this early state of T1DM only subjects with poor glycemic control exhibit advanced impairment of LV strain. The majority of the included patients either do not yet suffer from impaired contractility or are still in the previously described early occurring hyperdynamic state of diabetic cardiomyopathy. Taken together, our findings demonstrate the presence of early subclinical cardiac changes in diabetes mellitus that are most probably driven by metabolic dysfunction as previously suggested [73].

4.3. Type 1 Diabetic Children Have Signs of Beginning Diastolic Dysfunction. The present study provides further evidence for the presence of diastolic dysfunction in diabetes mellitus. We found a statistically significant decrease of E/A-ratio in poorly controlled T1DM patients when compared to healthy controls (see Figure 4). This is in accordance with observations in animal models [74–76] as well as with human MRI [64] and echocardiographic studies in pediatric [53] and adult [33, 77–80] patients with diabetes mellitus type 1 [33, 53, 80] and type 2 [77, 78] demonstrating signs of (beginning) diastolic dysfunction in nonischemic diabetic cardiomyopathy. A new aspect from the present study is the fact that signs of diastolic impairment already become evident very early in the course of T1DM. This further underlines the concept of nonischemic diabetes-associated myocardial impairment as a continuous process driven by metabolic imbalances.

4.4. Study Limitations. In a recent study on premature infants Sanchez and colleagues demonstrated a link of the reliability of two-dimensional speckle tracking derived deformation parameters and adjusted frame rate during image acquisition [81]. A frame rate/heart rate ratio of 0.7 to 0.9 frames per second per bpm has been proposed for optimal myocardial speckle tracking. In our study frame rate settings meet these criteria during echocardiography at rest. However, frame rates were not adjusted during stress testing. Accordingly, strain and strain rate parameters during exercise testing in our study may in fact be somewhat underestimated in both the study and the control group. Secondly, this was a cross-sectional study in a limited number of asymptomatic patients. Thus, the final clinical outcome of the observed subclinical alterations yet remains to be established in large study populations.

5. Conclusion

The present study provides further evidence for diabetes-associated nonischemic cardiomyopathy. A paradoxical increase of LV myocardial performance may occur very early in T1DM as a sign of impaired mechanical efficiency. T1DM patients with poor glycemic control have early signs of subclinical LV systolic and diastolic dysfunction. Consequently, tight glycemic control must be a high priority therapeutic aim for diabetic patients in order to minimize the ultimate risk of heart failure. Further experimental and clinical studies are needed in order to illuminate the spatiotemporal complexity of diabetes-associated heart failure.

Conflict of Interests

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

Authors' Contribution

Kai O. Hensel designed and supervised the study, interpreted the data, and wrote the paper. Franziska Grimmer performed the echocardiographic studies, postprocessing, and statistical analyses and prepared the figures and tables. Markus Roskopf was involved in echocardiographic image acquisition and postprocessing analyses. Andreas Heusch helped recruiting patients and performing echocardiographic examinations. Andreas Heusch, Stefan Wirth, and Andreas C. Jenke critically reviewed the paper.

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Review Article

Type 2 Diabetes and ADP Receptor Blocker Therapy

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Type 2 diabetes (T2D) is associated with several abnormalities in haemostasis *predisposing* to thrombosis. Moreover, T2D was recently connected with a failure in antiplatelet response to clopidogrel, the most commonly used ADP receptor blocker in clinical practice. Clopidogrel high on-treatment platelet reactivity (HTPR) was repeatedly associated with the risk of ischemic adverse events. Patients with T2D show significantly higher residual platelet reactivity on ADP receptor blocker therapy and are more frequently represented in the group of patients with HTPR. This paper reviews the current knowledge about possible interactions between T2D and ADP receptor blocker therapy.

1. Introduction

Type 2 diabetes (T2D) is associated with several abnormalities in haemostasis, such as higher platelet reactivity [1, 2], endothelial dysfunction [3], and hypercoagulation and abnormalities in fibrinolysis [4], predisposing to thrombosis. ADP receptor blocker therapy is crucial in acute coronary syndrome (ACS) and postpercutaneous coronary intervention (PCI) patients to prevent future thrombotic events. According to current European Society of Cardiology and American Heart Association Clinical Practice Guidelines [5–7] ADP receptor blocker therapy should be administrated in all ST-elevation myocardial infarction (STEMI) and non-ST-elevation myocardial infarction (NSTEMI)/unstable angina (UA) patients, while in STEMI patients undergoing primary PCI new ADP receptor blockers (prasugrel, ticagrelor) should be preferred; in patients with NSTEMI/UA prasugrel should be used just when coronary anatomy is already known and a decision to perform PCI has been already established. Otherwise, ticagrelor or clopidogrel should be administrated. Moreover, these recommendations should be fully applicable in patients with as well as without T2D. Nevertheless, T2D was recently associated with a failure in antiplatelet response to clopidogrel [8, 9] which remains the most commonly used ADP receptor blocker in clinical practice [10]. Importantly, clopidogrel high on-treatment platelet reactivity (HTPR) was consistently associated with the risk of ischemic adverse events. This paper reviews the current approaches of ADP receptor blocker therapy in T2D patients.

2. Clopidogrel and Its Resistance in T2D Patients

Thienopyridine clopidogrel is an oral irreversible P2Y12 ADP receptor blocker. This prodrug requires oxidation by the hepatic cytochrome P450 system to generate an active metabolite. After absorption, an estimated 85% of the prodrug is hydrolysed by esterases into an inactive form, leaving only 15% of clopidogrel available for transformation to the active metabolite, which irreversibly and selectively inactivates P2Y12 ADP receptor and inhibits ADP-induced platelet aggregation [11]. The introduction of clopidogrel by the CURE study in patients with ACS [12] significantly improved the clinical outcome compared with patients treated with aspirin alone. Similar outcome was subsequently obtained in post-PCI patients [13, 14]. However, the antiplatelet effect of clopidogrel varies among individuals.

Table 1: ADP receptor blockers in current clinical practice.

Drug	Route of administration	Bioavailability	Receptor inhibition	Time to peak platelet inhibition	Clinical application	Interactions with T2D
Clopidogrel	Oral	Prodrug	Irreversible	Highly variable	PCI, arterial interventions, ACS, stroke, and secondary prevention	Repeatedly proven
Prasugrel	Oral	Prodrug	Irreversible	2 hours	ACS with PCI	Not explicitly proven
Ticagrelor	Oral	Direct-acting	Reversible	2 hours	ACS	Probably none
Cangrelor	Intravenous	Direct-acting	Reversible	30 minutes	PCI	Not studied

ACS: acute coronary syndromes, PCI: percutaneous coronary intervention, T2D: type 2 diabetes.

As mentioned previously, there are a growing number of data pointing to the failure in antiplatelet responses to clopidogrel which is specifically associated with insulin resistance and T2D [8, 9, 15]. These reports are based on ex vivo testing of platelet reactivity on clopidogrel therapy, as well as on subanalysis of clinical trials with clopidogrel. In these trials patients with T2D on clopidogrel therapy had worse clinical course and increased incidence of stent thrombosis [8, 9, 15–19]. The exact mechanism of this phenomenon remains currently unknown. However, the mechanism of poor clopidogrel response in T2D patients is probably multifactorial. T2D per se increases the platelet reactivity to ADP. Insulin could reduce the platelet aggregation by inhibiting the P2Y12 pathway through insulin receptors [20]. Insulin resistance might upregulate the P2Y12 ADP receptor, which is associated with clopidogrel resistance [21, 22]. An absolute or a relative lack of insulin was previously associated with increased P2Y12 signalling capacity. Moreover, this pathway appears to be in patients with T2D less sensitive to P2Y12 inhibition [23]. On the other hand, T2D may also interact with clopidogrel metabolism. T2D is already known to modulate cytochrome P450 activity in humans and in animal models [24–26]. Erlinge et al. [8] studied the prevalence and mechanism of antiplatelet failure to clopidogrel in T2D patients and in nondiabetic individuals. This double blinded study randomized totally 110 patients already treated with aspirin to clopidogrel (600 mg loading dose followed by a maintenance dose of 75 mg) or prasugrel (60 mg loading dose followed by daily maintenance dose of 10 mg) for a period of 28 days. Results of the study showed significantly higher incidence of HTPR in patients treated with clopidogrel compared to prasugrel. Diabetic patients were more frequently represented in the group with HTPR. Moreover, the HTPR was in T2D patients connected to the administration of clopidogrel. When compared with nondiabetic patients, patients with diabetes had significantly lower concentrations of clopidogrel active metabolite measured two hours after a loading dose administration ($p < 0.01$) and also on 29th day of maintenance dose usage ($p < 0.01$). It is interesting that, in this study, platelets of diabetic patients with HTPR responded well to ex vivo administration of the active clopidogrel metabolite. This observation indicates a low level of resistance on platelet P2Y12 ADP receptor and supports a potential interaction between T2D and pharmacokinetic processes of clopidogrel metabolism.

Angiolillo et al. [9] studied platelet function in diabetic and nondiabetic patients treated with aspirin and clopidogrel. Blood samples were taken after loading dose administration and on chronic therapy. The authors found significantly higher residual platelet reactivity in T2D patients both prior to clopidogrel administration and 24 hours after clopidogrel loading dose administration. In addition, the authors found a significantly higher number of patients with clopidogrel HTPR among patients with T2D. It is already known that HTPR is an independent predictor of cardiovascular events [9] and platelet reactivity on clopidogrel therapy higher than 50% was repeatedly associated with higher risk of coronary events after PCI [17, 18, 27].

The worse clinical outcome and an increased risk of ischemic events in clopidogrel-treated T2D patients were consistently demonstrated in the subanalysis of the CURE [12], CREDO [28], and Current-OASIS 7 [29] trials. These data indirectly support an incomplete response to clopidogrel associated with T2D. Additionally, Iakovou et al. [19] in an analysis of data from a prospective observational study showed that T2D is an independent predictor of stent thrombosis, despite dual antiplatelet therapy in patients after successful implantation of drug eluting stents. High frequency of clopidogrel HTPR led to the introduction of new ADP receptor blockers with more favourable pharmacodynamic profile to clinical practice.

3. Prasugrel: New ADP Receptor Blocker in T2D Patients

Prasugrel (Table 1) is a new thienopyridine P2Y12 ADP receptor blocker, recently introduced to clinical practice in patients with ACS and planned PCI. Prasugrel compared to clopidogrel offers more consistent inhibition of P2Y12 ADP receptor and has a lower intraindividual variability in efficacy. Prasugrel was extensively tested in the TRITON-TIMI 38 trial [30] which randomized 13 608 patients with ACS to clopidogrel or prasugrel. These patients were treated from 6 to 15 months. In this trial 3146 of patients had T2D; 776 patients were treated with insulin. The primary "endpoint" of this study was significantly decreased by prasugrel in nondiabetic group (9.2%

versus 10.6%, $p < 0.05$), as well as in those with T2D (12.2% versus 17.0%, $p < 0.001$). Benefit of prasugrel administration was observed consistently in insulin-treated patients (14.3% versus 22.2%, $p < 0.01$), as well as in T2D patients without insulin therapy (11.5% versus 15.3%, $p < 0.01$). Prasugrel significantly reduced the incidence of myocardial infarction (MI) by 18% in nondiabetic subjects and by 40% in subjects with T2D. Moreover, this study showed a significant reduction of stent thrombosis by prasugrel in the overall group (0.9% versus 2.0%), as well as in T2D patients (2.0% versus 3.5%). Nevertheless, major bleeding events not associated with coronary artery bypass graft surgery occurred overall significantly more often in patients treated with prasugrel, compared to clopidogrel (2.4 versus 1.8%). In summary, throughout the study, the greatest benefit of prasugrel therapy was observed preferentially in T2D patients, in whom prasugrel significantly reduced the risk of ischemic events, including the risk of recurrent MI and the risk of stent thrombosis, without increasing the risk of serious bleeding.

On the other hand, the efficacy of prasugrel is not so convincing in patients who do not undergo invasive coronary revascularization. The TRIOLOGY ACS study [31]—a double blind, randomized prospective trial involving 7243 patients—failed to proof the significant reduction of the primary endpoint with prasugrel (10 mg daily) compared to clopidogrel (75 mg daily). Similar bleeding risk was observed in both groups of patients. In this study, 37.7% of prasugrel-treated and 38.3% of clopidogrel-treated patients had a history of T2D. Although the subanalysis of T2D patients was not reported specifically, generally there was no significant difference in the hazard ratio for primary endpoint in T2D patients compared to nondiabetic individuals (17.8% versus 11.5% in clopidogrel-treated patients, 20.4% versus 13.2% in prasugrel-treated patients, resp.; $p = 0.71$). Nevertheless, in this study, reduced ADP blocker loading doses (30 mg of prasugrel and 300 mg of clopidogrel) were administrated only in patients who underwent randomization within first 72 hours after the first medical contact and were not previously pretreated with ADP receptor blocker. Patients who did not undergo randomization within first 72 hours were treated with daily maintenance dose administration (i.e., loading dose was not administrated). This fact could influence the reduction of the primary endpoint of this study.

4. Prasugrel Resistance: A New Phenomenon in Diabetic Patients with ACS?

Prasugrel was repeatedly described as an effective drug for overcoming clopidogrel resistance [27, 32]. However, several recently published data reported an incomplete response to prasugrel. Prasugrel resistance might therefore become another problem in patients requiring ADP receptor blocker therapy. Silvano et al. described a rare case of resistance to both clopidogrel and prasugrel in nondiabetic patient with acute STEMI due to stent thrombosis [33]. In addition, results of recently published studies [34, 35] suggest that real prevalence of HTPR in prasugrel-treated patients may be higher than that which is traditionally considered. Bonello et al. [35] pointed out the fact that up to 25% of patients with ACS did not reach effective antiplatelet response even after 6–12 hours from prasugrel loading dose administration. There is no definite answer to the question of a possible relationship between T2D and the phenomenon of "prasugrel resistance." We have previously described a delayed antiplatelet response to prasugrel in two T2D patients undergoing primary PCI for acute STEMI [36]. Consequently, Alexopoulos et al. [37] reported in an observational study involving 77 patients with ACS undergoing PCI that platelet reactivity in prasugrel-treated patients differed significantly by T2D status. By multivariable analysis, insulin-treated T2D was identified as the only predictor of high platelet reactivity ($p < 0.01$). The authors concluded that patients with insulin-treated T2D treated with prasugrel post-PCI have higher platelet reactivity than patients without T2D or noninsulin-treated diabetic patients. This observation supports the possible interaction between T2D and prasugrel HTPR. However, this possible interaction remains inadequately explained and further studies will be needed for the final clarification of this issue.

5. Cangrelor: The New Member of the ADP Receptor Blockers Family

Cangrelor (Table 1) is an intravenously administrated adenosine triphosphate analogue that binds reversibly and with high affinity to P2Y12 ADP receptor. It offers a highly effective inhibition of ADP-induced platelet aggregation immediately after administration and allows the restoration of platelet function within 1-2 hours of its discontinuation [38]. Cangrelor has been investigated in three clinical trials including a total of 24 910 patients [39–41]. A meta-analysis of these studies [42] observed a 19% risk reduction rate in periprocedural death, MI, ischemia-driven revascularization, and stent thrombosis, with a 39% risk reduction rate in stent thrombosis alone. The TIMI major and minor bleeds were increased, but there was no increase in the rate of transfusions. This new agent may be considered in ADP receptor blocker naïve patients undergoing PCI for ACS [6]. Recently, there is no study specifically investigating possible interactions between T2D and antiplatelet response to cangrelor.

6. Ticagrelor: A Safe and Effective ADP Receptor Blocker in T2D Patients?

Ticagrelor (Table 1) is a new oral, direct reversible P2Y12 ADP receptor blocker which achieves a higher range of inhibition of platelet aggregation compared to clopidogrel [43]. The PLATO study [44] tested the efficacy of ticagrelor and clopidogrel in the prevention of cardiovascular events in patients with ACS (totally 18.624 patients enrolled). The incidence of the primary endpoint after 12 months of follow-up was significantly lower in patients treated with ticagrelor (10.2% versus 12.3%, $p < 0.001$); there was also a significant reduction of cardiovascular deaths and stent thrombosis in the subgroup of ticagrelor-treated post-PCI patients. Ticagrelor administration was not associated with an increased risk of serious bleeding. In the group of diabetic patients ticagrelor reduced the incidence of the primary endpoint, all-cause mortality, and the risk of stent thrombosis. Similar benefit of

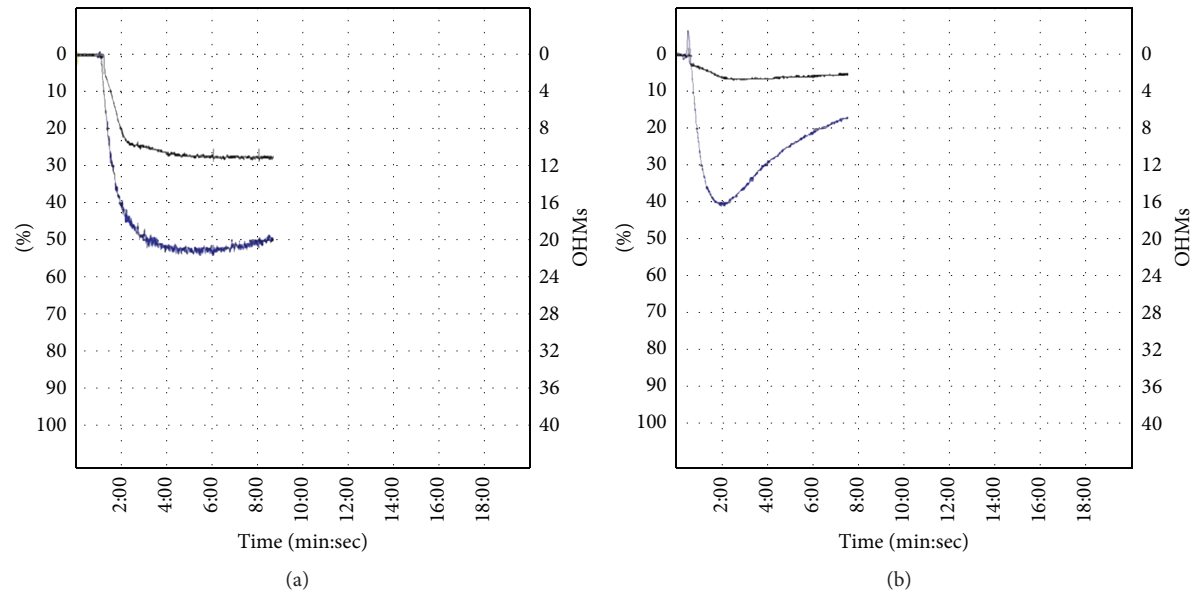

Figure 1: LTA with specific inducers (arachidonic acid: black curve, adenosine diphosphate: blue curve) showing difference between HTPR (a) and sufficient antiplatelet response (b) in T2D patient with acute ST-elevation myocardial infarction.

ticagrelor therapy was seen in insulin-treated T2D patients, as well as in diabetic patients without insulin therapy. In addition, Alexopoulos et al. [45] showed significantly lower platelet reactivity in ticagrelor-treated T2D patients compared to T2D patients treated with prasugrel. Moreover, in this single-center prospective randomized study none of the T2D patients was identified as a nonresponder for ticagrelor. Consistently, ticagrelor treatment was demonstrated to be effective and even superior to prasugrel [46] in high risk diabetic patients with ACS. These data suggest that ticagrelor may be a safe and effective ADP receptor blocker in T2D patients, which can ensure consistent platelet inhibition, without the risk of HTPR, together with a good safety profile.

7. Detection of HTPR in Clinical Practice

Assessing the individual level of platelet inhibition by implementing platelet function testing might help to identify patients with HTPR and therefore to reduce ischemic events. To assess the predictive level of platelet reactivity on ADP receptor blockers, numerous platelet function tests are currently available. Light transmission aggregometry (LTA) with specific inducer (adenosine diphosphate (ADP)) represents nowadays a "golden standard" in antiplatelet response testing. Maximal aggregation in response to ADP with LTA testing > 50% (Figure 1) had been associated with higher risk of ischemic events [47]. Second, vasodilator-stimulated phosphoprotein (VASP) phosphorylation flow cytometry assay represents a specific method for the assessment of ADP receptor blocker activity [48]. We have previously demonstrated that this assay is suitable for monitoring the ADP receptor blocker therapy in acute STEMI patients with primary PCI of culprit coronary lesion [49]. The advantage of this assay is its specificity for ADP receptor intracellular signaling pathway and sample stability. Nevertheless, instrumental and financial requirements may represent a possible limitation for the application of this assay in clinical practice. Third, several point-of-care assays are recently available. PFA-100 (Siemens Healthcare Diagnostics, Tarrytown, New York, USA) and Verify Now (Accumetries, San Diego, California, USA) assay methods—both based on modified aggregometry—allow quick platelet function testing in the setting of the intensive care units. Verify Now allows rapid assessment of platelet response on aspirin, P2Y12 ADP receptor antagonist, and glycoprotein IIb/IIIa antagonist treatment in one blood sample [50]. Bed site ADP receptor blockers testing may provide a rough guiding on how to proceed with treatment drugs and dosages, especially when both LTA and VASP phosphorylation assays are not available.

Although monitoring of ADP receptor blocker therapy is nowadays not generally recommended, this testing can significantly help to identify patients with HTPR. On the other hand, recently there is no definite answer to the question whether HTPR is a modifiable phenomenon. Several randomized studies trying to overcome HTPR with modified clopidogrel therapy guided by platelet function testing [51, 52] brought negative results. However, new antiplatelet agents were rarely used in these trials. Modified (increased) clopidogrel dosing, which was mostly used in these trials for overcoming the HPTR, failed to reduce the rate of major adverse cardiac events (cardiovascular death, nonfatal myocardial infarction, or stent thrombosis). The results of these randomized studies predominantly do not support a treatment strategy of high-dose clopidogrel in patients with HTPR and question the need of monitoring the on-treatment platelet reactivity in clinical practice. Nevertheless, a recently published observational study, which tested patients with planned PCI for stable angina or NSTE ACS [53], showed

a reduced risk of adverse clinical events in HTPR patients with tailored intensified antiplatelet therapy. Thus, monitoring and tailoring the antiplatelet therapy might be beneficial in selected patients and deserve further investigation.

In summary, T2D seems to be associated with HTPR especially in clopidogrel-treated patients. Moreover, we have previously confirmed the association between HTPR and stent thrombosis in post-PCI patient with T2D [27]. Therefore, it is probably reasonable to routinely prefer new ADP receptor blockers over clopidogrel in T2D patients in order to ensure more effective platelet inhibition and prevent these serious thrombotic adverse events. Additionally, the subanalysis of T2D patients treated with new ADP receptor blockers did not reveal higher risk of serious bleeding. This indicates that the benefit/risk ratio is in favour of new antiplatelet agents. In case of choosing clopidogrel therapy in T2D patients, it seems to be reasonable to perform platelet function testing for the approval of sufficient on-treatment response. If this response is inadequate, the switch to new ADP receptor blocker therapy should be considered immediately. In addition, ticagrelor, in T2D patients with ACS, was demonstrated as more effective and superior even to prasugrel [46]. Thus this agent should be preferred especially in case of diabetics with acute coronary events. Nevertheless, the higher cost of medication, patient compliance, higher risk of bleeding, and other side effects should be also considered for a decision of ADP receptor blocker therapy strategy.

8. Conclusion

The above-mentioned evidence suggests that T2D is associated with clopidogrel HTPR. Patients with T2D show significantly higher residual platelet reactivity on clopidogrel therapy and are more frequently represented in the group of patients with clopidogrel HTPR. Moreover, several data reported that patients with insulin-treated T2D have higher residual platelet reactivity even on prasugrel therapy than patients without T2D or noninsulin-treated diabetic patients. On the other hand, ticagrelor treatment was demonstrated to be effective and even superior to prasugrel in high risk diabetic patients with ACS and ticagrelor may be a safe and effective ADP receptor blocker in these patients. However, the relationship between T2D and ADP receptor blocker therapy is not fully explained and deserves further investigation.

Disclosure

Formal consent for this type of study is not required.

Conflict of Interests

The authors have no conflict of interests to declare.

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Research Article

Resting Heart Rate Does Not Predict Cardiovascular and Renal Outcomes in Type 2 Diabetic Patients

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Elevated resting heart rate (RHR) has been associated with increased risk of mortality and cardiovascular events. Limited data are available so far in type 2 diabetic (T2DM) subjects with no study focusing on progressive renal decline specifically. Aims of our study were to verify RHR as a simple and reliable predictor of adverse disease outcomes in T2DM patients. A total of 421 T2DM patients with variable baseline stage of diabetic kidney disease (DKD) were prospectively followed. A history of the cardiovascular disease was present in 81 (19.2%) patients at baseline, and DKD (glomerular filtration rate < 60 mL/min or proteinuria) was present in 328 (77.9%) at baseline. Progressive renal decline was defined as a continuous rate of glomerular filtration rate loss ≥ 3.3% per year. Resting heart rate was not significantly higher in subjects with cardiovascular disease or DKD at baseline compared to those without. Using time-to-event analyses, significant differences in the cumulative incidence of the studied outcomes, that is, progression of DKD (and specifically progressive renal decline), major advanced cardiovascular event, and all-cause mortality, between RHR </≥65 (arbitrary cut-off) and 75 (median) bpm were not found. We did not ascertain predictive value of the RHR for the renal or cardiovascular outcomes in T2DM subjects in Czech Republic.

1. Introduction

Elevated resting heart rate (RHR) has been associated with increased risk of all-cause mortality and cardiovascular (CV) events in healthy subjects as well as those with preexisting CV disease (CVD) including hypertension, acute myocardial infarction, and heart failure or left ventricular dysfunction by numerous epidemiologic studies and recently reviewed ones by Palatini and Julius [1] and Fox et al. [2]. In a recent study of Woodward et al., individual data from 112,680 subjects in 12 cohort studies were collected and an association between RHR above 65 beats/min (bpm) and the risk of both CV and all-cause mortality has been found independent of preexisting CVD [3]. Plausible pathophysiological mechanisms were reviewed by Lang et al. [4] and include, briefly, both indirect mechanisms related to autonomic dysregulation and those directly related to an increased heart rate per se (such as increased ischaemic burden and local haemodynamic forces adversely impacting on the endothelium and arterial wall).

Several studies focused on RHR in type 2 diabetic (T2DM) subjects. Stettler et al. found an association between RHR and all-cause mortality and CVD in a cohort of 302 T2DM patients [5]. Linnemann and Janka have identified an elevated RHR as a high risk for CV death in a cohort of 475 T2DM patients [6]. Hillis et al. found a relationship between baseline higher RHR and all-cause mortality, CV death, and major CV events (nonfatal myocardial infarction or nonfatal stroke) in a cohort of 11,140 T2DM patients; the increased

risk associated with a higher baseline RHR was most obvious in patients with previous macrovascular complications [7]. Hillis et al. also extended the study on the same cohort of T2DM patients on the effect of RHR and microvascular complications (nephropathy and retinopathy) and reported an increased incidence and a greater progression of [8].

There are, however, fewer data on the relationship of RHR and renal events in diabetic subjects. Miot et al. studied a cohort of 1088 T2DM patients for the association of RHR with the incidence of composite CV and renal endpoint (CV death, nonfatal myocardial infarction and/or stroke, hospitalization for heart failure, and renal replacement therapy) and also for the renal endpoint alone. While in patients without CVD no relationship was found, in the subgroup with CVD history at baseline significant association between RHR and the incidence of CV and/or renal events was ascertained [9]. However, "hard" renal end-point, an end-stage renal disease (ESRD), is impractical in majority of observational cohorts and interventional studies due to relatively short follow-up. Furthermore, diabetic kidney disease (DKD) appears to be phenotypically heterogeneous (see further) and thus pathways and mediators (e.g., RHR) leading to ESRD might differ.

As documented by recent studies in both types of diabetes, progressive renal decline (defined as continuous rate of glomerular filtration rate (GFR) loss ≥ 3.3% per year) might coexist with a "classical" form of DKD with increased urinary albumin excretion preceding GFR decline [10–12]. No study, so far, focused on predictive power of RHR for DKD progression considering both phenotypes (albuminuric versus nonalbuminuric DKD) in T2DM patients.

Therefore, the aims of the present study were (1) to evaluate whether RHR is associated with DKD stage or CVD at baseline, (2) to eventually replicate in our cohort of T2DM patients previous sporadic positive findings on RHR as a predictor of CVD and DKD endpoints and death in T2DM patients, and finally (3) to specifically address RHR predictive potential for progressive renal decline in our cohort.

2. Materials and Methods

2.1. Subjects. A total of 421 T2DM patients (unrelated Caucasian subjects from South Moravia region, Czech Republic), 51.5% of men, with median age 67 [IQR 61–75], median DM duration 14 years [IQR 8–21], and range of DKD stages at baseline, were enrolled into the study between 2002 and 2010. Prospective data were collected until 2013.

Severity of DKD was defined according to the urinary albumin excretion (UAE) and stage of chronic kidney disease (CKD) by GFR assessed by creatinine clearance based on 24 h urine collection. Both parameters, UAE and GFR, were repeatedly measured at least once in 6 months or more often; staging for DKD and CKD was based on two consecutive values. At baseline, the study sample consisted of normoalbuminuric subjects (UAE < 30 mg/24 h, 8.8%), microalbuminuric subjects (UAE 30–300 mg/24 h, 30.4%), macroalbuminuric subjects (UAE > 300 mg/24 h, 51.5%), and subjects with end-stage renal disease (ESRD, 9.3%). Respective staging for CKD in the same sample was CKD I (GFR ≥ 90 mL/min per 1.73 m^2, 17.3%), CKD II (60–89 mL/min per 1.73 m^2, 18.3%), CKD III (30–59 mL/min per 1.73 m^2, 36.9%), CKD IV (15–29 mL/min per 1.73 m^2, 16.3%), and subjects with CKD V at baseline (GFR < 15 mL/min per 1.73 m^2 or maintenance haemodialysis, 11.2%). Progressive renal decline was defined as a negative change of GFR equal to or steeper than 3.3% per year and the patient is referred to as a "decliner" and the rest as "nondecliners." Cut-off of GFR loss ≥ 3.3% per year has been used in previous reports [10, 11] and corresponds to the 2.5th percentile of the distribution of annual renal function loss in a general population [13]. A history of DKD at baseline (DKD-b^+) was defined as GFR < 60 mL/min/1.73 m^2 or macroalbuminuria. A history of CVD at baseline (CVD-b^+) was defined as a history of coronary artery disease, nonfatal myocardial infarction or stroke, lower limb amputation, or revascularization. RHR at baseline was determined either by 1 minute radial artery palpation or from ECG records. For detailed description of the whole group and CVD-b^+ and CVD-b^- or DKD-b^+ and DKD-b^- subgroups see Table 1.

Informed consent was obtained from each patient prior to being included in the study. The study was performed according to the recommendations of the Declaration of Helsinki and approved by the Ethical Committee of Medical Faculty, Masaryk University Brno.

2.2. Follow-Up. Subjects were prospectively followed for a median of 43 [22–77] months in diabetes and nephrology units of the two university hospitals in Brno and following end-points were considered as (1) progression of DKD defined as a decline of GFR < 60 mL/min per 1.73 m^2 during the follow-up period for those with GFR ≥ 60 mL/min per 1.73 m^2 at baseline or achieving ESRD, development of overt macroalbuminuria in normo- and microalbuminuric subjects at baseline, or progression of CKD by at least stage for those with CKD III and IV at baseline, (2) major adverse cardiovascular event (MACE), that is, fatal or nonfatal myocardial infarction or stroke, lower limb amputation, or revascularization, and (3) all-cause mortality (ACM). Only non-ESRD/non-CKD V at inception patients with complete follow-up information were included in the time-to-event analysis; that is, a total of 376 subjects were considered with the 48 month follow-up median [28–79].

2.3. Statistical Analysis. Data are expressed as median [interquartile range, IQR] or as percentages. Differences in continuous variables between the groups were analysed using Mann-Whitney test. Kaplan-Meier curves with log-rank testing were applied for time-to-event analysis to analyse the effect of RHR categories (< and ≥ actual median RHR and arbitrary cut-off 65 bpm) on studied outcomes. Standard competing risk methodology focusing on cumulative incidence was adopted for the nonparametric estimation and modelling of associations of the potential risk factors and the progression of DKD, MACE, and death. Gray test [14] was used to assess the differences in cumulative incidence of the competing risks with respect to the risk factors, and Fine and Gray model [15] was used to evaluate the predictive potential of the considered parameters. For all standard analysis,

Table 1: Clinical and biochemical characteristics of subjects divided according to history of CVD, baseline data.

Variables	All	CVD-b$^+$	CVD-b$^-$	P
n (%)	421	81 (19.2)	340 (80.0)	
Sex: men/women, n (%)	217 (52)/204 (48)	52 (64)/29 (36)	165 (49)/175 (51)	0.01
Age (years)	67 [61–75]	70 [63–76]	67 [60–74]	0.009
Diabetes duration (years)	14 [8–21]	16 [12–23]	13 [7–20]	1×10^{-4}
FPG (mmol/L)	8.5 [6.8–10.9]	9.3 [7.8–11.8]	8.1 [6.6–10.8]	0.02
HbA1c (%), IFCC calibration	6.4 [5.4–8.1]	6.6 [5.9–8.0]	6.4 [5.2–8.1]	NS
Creatinine (μmol/L)	142 [114–214]	188 [139–311]	135 [101–191]	$<1 \times 10^{-6}$
Urea (mmol/L)	11.0 [7.3–17.3]	16.0 [10.1–21.6]	9.9 [6.9–16.2]	$<1 \times 10^{-5}$
Albuminuria (mg/24 hours)	500 [140–2080]	1540 [350–3670]	400 [130–1740]	7×10^{-4}
Systolic blood pressure (mmHg)	144 [130–160]	145 [125–150]	142 [130–160]	NS
Diastolic blood pressure (mmHg)	80 [75–90]	80 [70–95]	80 [75–90]	NS
Total cholesterol (mmol/L)	4.9 [4.2–5.8]	4.5 [3.9–5.3]	4.9 [4.3–5.8]	0.02
Beta blockers users (%)	46.3	50.6	45.3	NS
RAAS inhibitors users (%)	62	53.1	64.1	NS
Other antihypertensive therapy (%)	69.1	71.6	68.5	NS
History of renal disease, n (%)	**328 (77.9)**	**73 (90)**	**255 (75)**	**NS**
Decliners (GFR loss ≥ 3.3% per year), n (%)	**191 (45.4)**	**36 (44)**	**155 (45.6)**	**NS**
RHR (bpm)	75 [70–80]	75 [70–84]	75 [70–80]	NS

Data are expressed as median [interquartile range] or percentages. Differences evaluated by nonparametric Mann-Whitney or chi-square test, respectively. FPG, fasting plasma glucose; HbA1c, glycated haemoglobin; GFR, glomerular filtration rate; RAAS, renin-angiotensin-aldosterone system; RHR, resting heart rate.

Statistica for Windows (Statsoft Inc., Tulsa, OK, USA) was used. $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Analysis of Baseline Data. A history of the CVD was present in 81 (19.2%) patients at baseline; DKD (GFR < 60 mL/min or proteinuria) was present in 328 (77.9%) at baseline. For characteristics of subjects, see Tables 1 and 2.

In a CVD-b$^+$ subgroup of patients (groups divided according to having or not having CVD at baseline), they were more frequently men, significantly older, and with a longer diabetes duration, higher fasting plasma glucose (FPG) levels, higher urea and creatinine plasma levels, and higher degree of albuminuria, while they did not differ in RHR, systolic (SBP) and diastolic (DBP) blood pressure, and HbA1c levels compared to CVD-b$^-$ subgroup. Finally there was no significant difference in the presence of a renal history at baseline and a proportion of decliners between subgroups.

When the patients were divided according to having or not having DKD at baseline, DKD-b$^+$ subjects had (similarly to CVD-b$^+$) higher age, longer diabetes duration, higher urea and creatinine plasma levels and higher degree of albuminuria, additionally higher DBP and higher frequency of CVD at baseline, and finally higher proportion of decliners. There was no difference in sex, FPG, HbA1c levels, SBP, and again RHR.

Comparisons of possible differences in RHR in the presence or absence of the treatment with beta-blockers, renin-angiotensin-aldosterone system blockers (i.e., sartans or angiotensin 1 receptor, 2 blockers), or other antihypertensive therapy (i.e., diuretics, Ca-blockers, and central antihypertensive drugs) revealed no statistically significant differences in the whole group or any of the CVD-b$^{+/-}$ or DKD-b$^{+/-}$ subgroups (all $P > 0.05$, Mann-Whitney test). For frequencies of each drug group prescription within the whole group or subgroups, see Tables 1 and 2 (all $P > 0.05$, chi-square test).

Finally, we assessed correlations of RHR with other clinical data (age, diabetes duration, SBP, DBP, FPG, HbA1c, creatinine, urea, and total cholesterol) and found significant correlations with FPG ($r = 0.12$, $P = 0.02$, Spearman) and SBP ($r = 0.16$, $P = 0.028$, Spearman).

3.2. Follow-Up Analysis

(A) Kaplan-Meier Analysis of Separate End-Points. During the follow-up period, cumulative incidences of DKD progression, MACE, and all-cause mortality were 48.3%, 23.1%, and 38.9%, respectively, in the whole group; for incidences in CVD-b$^{+/-}$ or DKD-b$^{+/-}$ subgroups separately, see Table 3. Of a total of 376 subjects analysed in the follow-up study, 62 had both CVD and DKD at baseline. Of those, 35 (56%) died and 27 (44%) survived ($P > 0.05$, chi-square test). Median RHR was 75 bpm in the whole group. Furthermore, 191 (45.4%) of patients were found as GFR decliners. No statistically significant difference in RHR was ascertained between decliners and nondecliners ($P > 0.05$, Mann-Whitney test).

Irrespective of non-significant differences in RHR between subjects with CVD or DKD at baseline (b$^+$) compared to those without (b$^-$), analyses were still performed for (i) the whole group and (ii) CVD-b$^{+/-}$ and (iii) DKD-b$^{+/-}$

Table 2: Clinical and biochemical characteristics of subjects divided according to history of DKD, baseline data.

Variables	All	DKD-b$^+$	DKD-b$^-$	P
n (%)	421	328 (77.9)	93 (22.1)	
Sex: men/women, n (%)	217 (52)/204 (48)	175(53)/153 (47)	42 (45)/51 (55)	NS
Age (years)	67 [61–75]	68 [62–75]	63 [56–71]	3×10^{-4}
Diabetes duration (years)	14 [8–21]	15 [9–22]	10 [6–15]	4×10^{-5}
FPG (mmol/L)	8.5 [6.8–10.9]	8.8 [6.9–11.2]	8.0 [6.8–9.7]	NS
HbA1c (%), IFCC calibration	6.4 [5.4–8.1]	6.5 [5.5–8.1]	6.2 [5.3–8.0]	NS
Creatinine (μmol/L)	142 [114–214]	164 [125–258]	91 [81–107]	$<1 \times 10^{-6}$
Urea (mmol/L)	11.0 [7.3–17.3]	13.3 [8.7–19.6]	6.1 [5.3–7.6]	$<1 \times 10^{-6}$
Albuminuria (mg/24 hours)	500 [140–2080]	840 [260–2350]	110 [90–150]	$<1 \times 10^{-6}$
Systolic blood pressure (mmHg)	144 [130–160]	144 [130–160]	140 [130–160]	NS
Diastolic blood pressure (mmHg)	80 [75–90]	80 [74–90]	90 [80–98]	0.001
Total cholesterol (mmol/L)	4.9 [4.2–5.8]	4.9 [4.2–5.7]	4.9 [4.3–5.9]	NS
Beta blockers at treatment (%)	46.3	48.5	33.3	NS
RAAS inhibitors at treatment (%)	62	60.7	58.1	NS
Other antihypertensive therapy (%)	69.1	71	52.7	NS
History of CV disease, n (%)	**328 (77.9)**	**73 (22)**	**8 (9)**	**0.01**
Decliners (GFR loss ≥ 3.3% per year), n (%)	**191 (45.4)**	**164 (50)**	**27 (29)**	$\mathbf{3 \times 10^{-4}}$
RHR (bpm)	75 [70–80]	74 [70–80]	74 [70–80]	NS

Data are expressed as median [interquartile range] or percentages. Differences evaluated by nonparametric Mann-Whitney or chi-square test, respectively. CV, cardiovascular; FPG, fasting plasma glucose; HbA1c, glycated haemoglobin; GFR, glomerular filtration rate; RAAS, renin-angiotensin-aldosterone system; RHR, resting heart rate.

Table 3: Cumulative incidence of DKD progression, MACE and all-cause mortality in the whole group and subgroups.

	DKD progressor	P	MACE	P	ALL-cause mortality	P
Whole group ($n = 376$)	48.3%	—	23.1%	—	38.9%	—
CKD-b$^+$ ($n = 66$)	51.5%	NS	22.3%	NS	53.0%	NS
CKD-b$^-$ ($n = 310$)	47.1%		27.3%		36.1%	
DKD-b$^+$ ($n = 283$)	53.4%	0.047	26.8%	0.023	48.4%	$<1 \times 10^{-4}$
DKD-b$^-$ ($n = 93$)	26.9%		11.7%		6.5%	

subgroups. Using time-to-event analyses, any significant differences in the cumulative incidence of the three studied outcomes were found between RHR </≥75 bpm (i.e., our median) and 65 bpm (i.e., arbitrary cut-off used in majority of meta-analyses [3]) neither in the whole group nor in the CVD-b$^{+/-}$ or DKD-b$^{+/-}$ subgroups ($P > 0.05$, log-rank test).

In spite of the fact that RHR did not significantly differ between beta-blocker users and nonusers in the whole group or any of the subgroups defined based on CVD or DKD status at baseline, we still analysed the effect of RHR (cut-off </≥65 or 75 bpm) in beta-blocker naive subjects separately ($n = 145$). No significant differences were assessed using this subpopulation analysis (all $P > 0.05$, log-rank test).

(B) Competing Risk Analysis. Since estimates based on the naive Kaplan-Meier curves do not consider the presence of competing risks, they apparently tend to overestimate the probability of occurrence of the individual events in time. We compared groups with initial RHR </≥ median (75 bpm) or </≥ arbitrary cut-off (65 bpm), respectively. P values in univariate analysis of competing risk model did not indicate a significant effect of the RHR on the cumulative incidence of the two competing risks (i.e., DKD progression and MACE, $P > 0.05$, Gray test). Similarly, no significance was found for all-cause death ($P > 0.05$, Gray test). Table 4 shows the results of a Fine and Gray model. Again, no significant difference between groups was found.

4. Discussion and Conclusions

In the present study we evaluated a predictive potential of baseline RHR for progression of DKD (and more specifically for rapid GFR decline), MACE, and all-cause death in T2DM patients. In the cross-sectional part of our study, we compared clinical and biochemical data between groups of diabetic subjects with or without initial DKD or CVD. Subjects in both DKD-b$^+$ and CVD-b$^+$ subgroups had significantly higher age, longer diabetes duration, worse renal parameters (higher levels of urea and creatinine and higher degree of albuminuria), higher FPG, and male predominance in a CVD-b$^+$ subgroup, while DKD-b$^+$ subgroup had higher DBP and a higher proportion of decliners.

Although previous studies found an association between RHR and prevalence of baseline CVD [7–9] or DKD [8, 9] in

Table 4: (a) Fine and Gray model: the effect of patient and disease characteristics on the progression of DKD (univariate analysis). (b) Fine and Gray model: the effect of patient and disease characteristics on the *MACE* (univariate analysis).

(a)

Risk factor	Risk category	Basal category	Fine and Gray model		
			HR	95% CI	*P* value
RHR	≥65 bpm	<65 bpm	1.11	0.77–1.60	0.580
RHR	≥75 bpm	<75 bpm	0.97	0.73–1.29	0.820

(b)

Risk factor	Risk category	Basal category	Fine and Gray model		
			HR	95% CI	*P* value
RHR	≥65 bpm	<65 bpm	0.79	0.51–1.23	0.300
RHR	≥75 bpm	<75 bpm	0.87	0.60–1.27	0.470

T2DM patients, we were not able to ascertain similar significant differences in RHR in any of those categories studied. The major focus of the study was the prospective evaluation of the predictive potential of RHR for the MACE and progression of DKD. Moreover, we believe, this is a first study dealing with RHR in relation to the progressive renal decline. The concept of progressive renal decline was proposed by Krolewski in T1DM patients as an alternative pathway to albuminuric DKD [16]. Pugliese et al. in the RIACE study on T2DM patients [12] found a reduced estimated GFR (eGFR) without albuminuria independently associated with a significant CVD burden, higher than albuminuria alone, whereas the combination of reduced eGFR and albuminuria marked a further increased risk of CVD events in an additive manner. We found a higher proportion of decliners in DKD-b^+ subgroup of patients, which could be explained by generally nonlinear pattern of renal disease progression; however, no such difference was found between CVD-b^+ and CVD-b^- subgroups in spite of the fact that CVD-b^+ group had worse renal parameters at baseline similarly to DKD-b^+. This might signify a specific pathogenic mechanism unrelated to CVD and this topic warrants further study.

Since beta-blockers or RAAS blockers have an obvious influence on RHR, we adjusted our analyses for the therapy modality. There was no therapy-related effect on any of the outcomes studied and on any of subgroups.

Our finding of positive correlation of RHR with FPG and SBP corresponds with results of previous studies; for example, in a large study of a French population with almost 100,000 participants, heart rate was positively associated with blood pressure, triglycerides, glycaemia, and physical inactivity and negatively with body height [17].

In the prospective part of the study, we were unable to identify any significant relationship of an initial RHR with DKD progression, major adverse cardiovascular event, and all-cause mortality in our cohort. Since more than one end-point may occur in the same patient, a competing risk methodology for multiple risk scenario was used. Yet again, RHR was not identified as a significant risk factor for DKD progression or MACE in the univariate competing risk model. Those findings are contrary to results of previous sporadic studies. A prospective study by Hillis et al. found in a cohort of 11,140 T2DM patients with a history of CVD participating in ADVANCE study [18] an association between higher baseline RHR and a greater risk of developing microvascular endpoint (defined as a composite of new or worsening nephropathy) during 4.4-year follow-up. After adjustment for age, sex, and randomized treatment (perindopril-indapamide), a 10 bpm increase in baseline RHR was associated with an 18% increase in the observed hazard [8]. Another recent study of 1088 T2DM patients by Miot et al. [9] focused on both CV and renal parameters (briefly, 31% of patients had a history of CVD at baseline (CVD-b^+) and median of follow-up was 4.2 years; mean RHR was 67.7 bpm in CVD-b^+ subgroup and 72.4 bpm in CVD-b^- subgroup) but not considering the drug therapy in the analyses ascertained RHR associated with the incidence of CV and renal morbidity/mortality ($P = 0.0002$) and also with renal risk alone adjusted for all-cause death as a competing event in the CVD-b^+ subgroup only ($P < 0.0001$). In the CVD-b^- subgroup, no relation was found between RHR and the incidence of CV and/or renal events. We have not been able to replicate any predictive effect of the RHR for the renal or CV outcomes in T2DM population of Czech Republic. Given similar settings of our study, one of the possible explanations might certainly be a smaller sample size, slightly shorter follow-up, different definition of endpoints, or different cut-offs for RHR. Regarding the latter, of plethora of possibilities, we have chosen stratification according to two RHR cut-offs, a median RHR and an arbitrary cut-off 65 bpm in line with results of a meta-analysis by Woodward et al. [3].

There are several pathogenic mechanisms proposed by which an elevated heart rate might mediate development and progression of DKD and CVD. It has been suggested that a higher heart rate might promote microalbuminuria because of increased exposure of the glomerulus to arterial pressure waves [19]. An increased heart rate has also variety of direct detrimental cardiovascular consequences including endothelial dysfunction and atherogenic activity that are important factors in the progression of DKD too [20]. A higher heart rate also is associated with factors such as obesity, higher blood pressure, atherogenic dyslipidaemia, and reduced physical activity [21], all of which are associated with an increased risk of microvascular complications and

are targets for intervention to improve outcome in patients with diabetes mellitus [22]. Finally, a faster resting heart rate is a characteristic feature of autonomic neuropathy, which is in turn associated with an increased prevalence of other complications, such as DKD or retinopathy [23]. Therefore, it is conceivable that mechanisms listed could have synergistic effects and represent potentially very important pathogenic mechanism; on the contrary, the effect might operate in stage-dependent fashion given our negative finding of increased RHR as a general predictor of DKD progression in T2DM.

We are of course aware of several limitations of our study potentially impacting on its negative outcome. First of all, current sample size is relatively small compared to previous studies. This together with the rather high representation of subjects with baseline DKD or CVD might weaken the potential predictive power of RHR in the situation of more advanced stages of cardiovascularly relevant comorbidities. Therefore, although our results indicate several trends—for example, patients with a history of CVD or DKD at baseline had more frequently beta-blockers in therapy and CVD-b$^+$ patients have a tendency to a higher RHR—the results were not found statistically significant in our cohort.

In conclusion, recent study analysing the potential of RHR for the prediction of progression of DKD (and specifically progressive renal decline), major cardiovascular event, and all-cause death in a cohort of Caucasian T2DM subjects did not reveal significant effect (not even in the subgroup of heart rate affecting therapy-naïve subjects). Additional studies are therefore warranted to decipher event. Additional studies are therefore warranted to decipher if RHR could be an applicable risk marker for DKD.

Conflict of Interests

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

Authors' Contribution

Vendula Bartáková designed the study, analysed the data, and drafted the paper. Denisa Malúšková performed the statistical analysis. Veronika Dvořáková analysed the data. Jana Bělobrádková, Jitka Řehořová, Jindřich Olšovský, Linda Klimešová, Katarína Kianičková, and Jan Svojanovský contributed in recruiting participants and collecting demographic and anthropometric data. Kateřina Kaňková supervised the study and revised the paper. All authors read and approved the final paper.

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Research Article

Myocardial Perfusion and Function Are Distinctly Altered by Sevoflurane Anesthesia in Diet-Induced Prediabetic Rats

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Preservation of myocardial perfusion during surgery is particularly important in patients with increased risk for perioperative complications, such as diabetes. Volatile anesthetics, like sevoflurane, have cardiodepressive effects and may aggravate cardiovascular complications. We investigated the effect of sevoflurane on myocardial perfusion and function in prediabetic rats. Rats were fed a western diet (WD; $n = 18$) or control diet (CD; $n = 18$) for 8 weeks and underwent (contrast) echocardiography to determine perfusion and function during baseline and sevoflurane exposure. Myocardial perfusion was estimated based on the product of microvascular filling velocity and blood volume. WD-feeding resulted in a prediabetic phenotype characterized by obesity, hyperinsulinemia, hyperlipidemia, glucose intolerance, and hyperglycemia. At baseline, WD-feeding impaired myocardial perfusion and systolic function compared to CD-feeding. Exposure of healthy rats to sevoflurane increased the microvascular filling velocity without altering myocardial perfusion but impaired systolic function. In prediabetic rats, sevoflurane did also not affect myocardial perfusion; however, it further impaired systolic function. Diet-induced prediabetes is associated with impaired myocardial perfusion and function in rats. While sevoflurane further impaired systolic function, it did not affect myocardial perfusion in prediabetic rats. Our findings suggest that sevoflurane anesthesia leads to uncoupling of myocardial perfusion and function, irrespective of the metabolic state.

1. Introduction

Myocardial perfusion in relation to myocardial function determines the balance between myocardial energy supply and demand. During surgery, maintenance of myocardial oxygen balance is challenged. Extrinsic factors, like anesthetics and surgical stress, and intrinsic factors, such as cardiometabolic disease, affect myocardial oxygen supply and consumption. This altered balance may increase the vulnerability of the heart for an oxygen supply and demand mismatch and consequent ischemia [1, 2].

The volatile anesthetic sevoflurane exerts direct effects on the heart and circulation that jeopardise perioperative myocardial function and hemodynamic stability. Sevoflurane has vasodilating properties and is known to reduce coronary vascular resistance [3] and perfusion pressure [4, 5]. We showed that sevoflurane did not affect myocardial blood flow in cardiovascular healthy patients, while myocardial flow reserve was decreased [6]. Animal studies however showed that sevoflurane, when perfusion pressure remained constant, increased coronary blood flow in dogs [7] and decreased coronary flow reserve in isolated rat hearts [5]. In contrast, sevoflurane lowered blood pressure and decreased myocardial blood flow in healthy rats [8], dogs [9], and pigs [10].

While sevoflurane exerts contrasting effects on myocardial perfusion in healthy conditions, its vasodilatory impact may be more abundant in patients with cardiometabolic disease, like type 2 diabetes mellitus (T2DM). T2DM patients are more likely to develop coronary artery disease [11] and

have an increased cardiovascular complication rate after major noncardiac surgery [12]. Because myocardial substrate metabolism and myocardial oxygen balance are altered in T2DM [13], the regulation of myocardial perfusion in these patients is particularly important during intraoperative circumstances, such as hypoperfusion. We previously found that myocardial perfusion, but not myocardial function, is preserved during hyperemia in glucose intolerant rats [14], while others showed myocardial perfusion defects in diabetic insulin resistant patients [15, 16] and T2DM patients during the postprandial state [17, 18]. The number of studies focusing on myocardial perfusion during sevoflurane anesthesia in subjects with cardiometabolic disease is however limited. We recently showed that sevoflurane decreased myocardial blood flow in T2DM patients and also a trend towards a lower vasodilator capacity was observed [19]. Taken together, these data suggest that the anesthesia-related alterations in myocardial perfusion and function may be more prominent in the presence of cardiometabolic disease.

Therefore, the purpose of the present study was to investigate the additional effect of sevoflurane anesthesia on myocardial perfusion and function in diet-induced prediabetic rats. We hypothesized that the impact of sevoflurane anesthesia is more abundant in the presence of cardiometabolic disease and thereby challenges perioperative regulation of myocardial perfusion and function.

2. Materials and Methods

2.1. Animals and Experimental Setup. This study was carried out in strict accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. All experiments were approved by the Institutional Animal Care and Use Committee of the VU University (permit number ANES 12-04) and performed in compliance with the modern ARRIVE guidelines on animal research [20]. All surgeries were performed under S-Ketamine and diazepam anesthesia, and all efforts were made to minimize suffering.

The study was divided into two parts: (1) characterization of the phenotype induced by western diet feeding and (2) myocardial perfusion and function measurements during baseline conditions and sevoflurane exposure. The first part of the study was performed in a group of 16 male Wistar rats (baseline body weight: 264 ± 5 g; Charles River Laboratories, France), which were exposed to a western diet in combination with sucrose water (20%) (WD, $n = 8$) or control diet (CD, $n = 8$). After 8 weeks of diet exposure, rats underwent an oral glucose tolerance test. Rats were sacrificed after a 6 h fasting period by decapitation and trunk blood was collected for plasma determinations.

The second part of the study included 36 male Wistar rats (body weight 265 ± 7 g; Charles River Laboratories, France) that were exposed to either CD ($n = 18$) or WD ($n = 18$) as described above. After 8 weeks, rats underwent (contrast) echocardiography during baseline conditions and after 5 minutes of sevoflurane (2.0%) exposure.

All rats were housed in a temperature-controlled room (20–23°C; 40–60% humidity) under a 12/12 h light/dark cycle starting at 6.00 a.m. Body weight and caloric intake were determined on a weekly basis.

2.2. Diets. CD (Teklad 2016, Harlan, Horst, Netherlands) consisted of 20% kcal protein, 9% kcal fat, and 74% kcal carbohydrates (1804 kcal/kg starch, 200 kcal/kg sugars), whereas WD (D12451, Research Diets, New Brunswick, NJ) consisted of 20% kcal protein, 45% kcal fat, and 35% kcal carbohydrates (291 kcal/kg starch, 691 kcal/kg sugars) with 20% sucrose water (800 kcal/kg), totally containing 3300 kcal/kg and 4857 kcal/kg for CD and WD with sucrose water, respectively.

Part 1

2.3. Oral Glucose Tolerance Test. In the first part of the study, awake rats fasted overnight received an oral glucose load (2 g/kg of body weight). Blood glucose was measured from tail bleeds with a Precision Xceed Blood Glucose monitoring system (MediSense, UK) before (0) and 15, 30, 60, 90, and 120 min after glucose ingestion. At similar time points, plasma insulin (LINCO research, St. Charles, Missouri) levels were measured as described previously [14, 21].

2.4. Blood and Plasma Measurements. Plasma hematocrit levels were determined using microcentrifugation. Plasma glucose levels (Abcam, Cambridge, MA), plasma insulin (LINCO research, St. Charles, Missouri), plasma free fatty acids (WAKO NEFA-HR, Wako Pure Chemical Industries, Osaka, Japan), plasma triglyceride (Sigma, Saint Louis, Missouri), and plasma HDL and LDL/VLDL cholesterol (Abcam, Cambridge, MA) levels were measured from trunk blood as described previously [13, 14, 21, 22].

Part 2

2.5. Surgery. The rats in the second part of the study were anesthetized with 125 mg/kg S-Ketamine (Ketanest, Pfizer, Netherlands) and 4 mg/kg diazepam (Centrafarm, Netherlands) intraperitoneally. The trachea was intubated and lungs were mechanically ventilated (positive end-expiratory pressure, 1-2 cm H_2O; respiratory rate, ~65 breaths/min; tidal volume, ~10 mL/kg) with oxygen-enriched air (40% O_2/60% N_2). Anesthesia was maintained by continuous infusion of 50 mg/kg/h S-Ketamine and 1.3 mg/kg/h diazepam intravenously via the tail vein. Respiratory rate was adjusted to maintain pH and partial pressure of carbon dioxide within physiological limits. Body temperature was maintained stable (36.7 ± 1.2°C) using a warm water underbody heating pad.

A catheter was placed in the right jugular vein for infusion of the contrast agent. The left carotid artery was cannulated for blood sampling, blood gas analyses (ABL50, radiometer, Copenhagen, Denmark), and measurements of arterial blood pressure (Safedraw Transducer Blood Sampling Set, Argon Medical Devices, Texas, USA). Arterial blood pressure, ECG, and heart rate were continuously recorded using PowerLab software (PowerLab 8/35, Chart 7.0; ADInstruments Pty, Ltd., Castle Hill, Australia). Mean arterial blood pressure was calculated according the following formula: $2/3 \cdot$ diastolic blood pressure $+ 1/3 \cdot$ systolic blood pressure. Rate

pressure product (RPP) was calculated by the product of heart rate and systolic blood pressure and was used as an estimate of myocardial oxygen demand.

2.6. Preparation of Microbubbles. Microbubbles were prepared from perfluorobutane gas and stabilized with a monolayer of distearoyl phosphatidylcholine and PEG stearate. 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC; Avanti Polar Lipids, Alabama, USA) and polyoxyethylene stearate (PEG40; Sigma, St. Louis, MO, USA) were dissolved in glycerol (10 mg/mL) and sonicated (Decon FS200, Decon Ultrasonics Ltd., Sussex, UK) at 40 kHz in an atmosphere of perfluorobutane (F2 Chemicals Ltd., Lancashire, UK) and vials were shaken in a Vialmix at 4500 rpm (Bristol-Myers Squibb Medical Imaging, Massachusetts, USA). As the gas was dispersed in the aqueous phase, microbubbles were formed, which were stabilized with a self-assembled lipid/surfactant monolayer. Freshly made bubbles were then washed twice to remove excessive DSPC and PEG40 and stored refrigerated in sealed vials in perfluorobutane atmosphere. A Multisizer 3 Coulter Counter (Beckman Coulter Inc., Miami, FL, USA) was used to measure the particle size distribution as well as the number of particles. The average bubble concentration was $1.58 \cdot 10^9 \pm 0.36 \cdot 10^9$ and the particle range was between 1 and 10 μm. Microbubbles were diluted to a concentration of $200 \cdot 10^6$ with degassed NaCl.

2.7. Myocardial Contrast Echocardiography. After surgery (contrast) echocardiography was performed to determine myocardial function and perfusion during baseline conditions and after 5 minutes of sevoflurane (2%) exposure. Contrast echocardiography was performed using a Siemens (ACUSON, Sequoia 512) equipped with a 14 MHz linear array transducer [13, 14]. Microbubbles were continuously infused into the jugular vein with a rate of 300 μL/min using a dedicated syringe pump (Vueject, Bracco SA, Switzerland). After two minutes of microbubble infusion, perfusion images were taken from the long-axis view of the left ventricle.

Low acoustic power (mechanical index [MI] 0.20) was used for microbubble detection with a dynamic range of 50 dB. A perfusion sequence consisted of about 10 cardiac cycles of low MI imaging, followed by a burst of high acoustic power (MI 1.8) for complete contrast destruction. Subsequently, on average 20 cardiac cycles of low MI images were acquired to allow contrast replenishment in the myocardium. All data were stored for offline analysis.

2.8. Myocardial Contrast Echocardiography Analysis. Custom-designed software was used for analysis of the estimate of perfusion (Matlab, 7.10, R2010A, MathWorks Inc. Massachusetts, USA) [6, 14]. For each cardiac cycle, regions of interest were drawn in the end-systolic frame in the posterior wall in the long-axis view of the left ventricle. Myocardial signal intensities from the frames after microbubble destruction were corrected for background noise by subtracting the signal intensity of the first frame after microbubble destruction (Y_0). These intensities were then fitted ($Y = Y_0 + (A - Y_0) \cdot (1 - \exp^{(-\beta \cdot x)})$) for calculation of microvascular blood volume A and the microvascular filling velocity β, which corresponds to the capillary blood exchange rate. The estimate of perfusion was calculated as the product of A and β [23].

2.9. Echocardiography. Myocardial systolic function was determined with echocardiography [13, 14]. Briefly, left ventricular (LV) dimensions during end-systole (ES) and end-diastole (ED) were determined in the M- (motion-) mode of the parasternal short-axis view at the level of the papillary muscles. LV systolic function is represented by fractional shortening (FS) and fractional area change (FAC), which were calculated by the equations: $FS = (EDD - ESD)/EDD \cdot 100$ and $FAC = (EDD^2 - ESD^2)/EDD^2 \cdot 100$. All parameters were averaged over at least three cardiac contractile cycles.

2.10. Statistical Analysis. Data were analyzed using Graphpad Prism 5.0 (La Jolla, USA) and presented as mean ± SD. Between group comparisons (CD versus WD) were performed using a Student t-test, whereas the effect of sevoflurane was tested with a two-way ANOVA with Bonferroni as post hoc test. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Western Diet Feeding Resulted in a Prediabetic Phenotype. Eight weeks of western diet feeding resulted in a mild type 2 diabetic (prediabetic) phenotype with obesity, mild hyperglycemia, hyperinsulinemia, hyperlipidemia (Table 1), and glucose intolerance (Figure 1). Heart and liver weight and epididymal and perirenal fat pads were significantly increased in western diet-fed rats compared to controls. Furthermore, heart rate, systolic blood pressure, diastolic blood pressure, and mean arterial pressure remained unchanged (Table 1).

3.2. Impaired Myocardial Perfusion and Systolic Function in Prediabetic Rats. Compared to healthy controls, western diet feeding tended to decrease microvascular filling velocity (β) and significantly decreased microvascular blood volume (A), which resulted in a significant reduction in the estimate of perfusion (Figure 2).

Western diet feeding significantly increased end-systolic lumen diameter and diastolic wall thickness but did not affect end-diastolic lumen diameter and wall thickness during systole compared to control rats (Table 2). Fractional shortening and fractional area change were significantly decreased in western diet-fed rats compared to control animals, suggesting impaired systolic function (Figure 3).

3.3. Sevoflurane Further Impaired Systolic Function but Not Myocardial Perfusion in Prediabetic Rats. Blood pressure, heart rate, and rate pressure product were significantly decreased after 5 minutes of sevoflurane exposure and significantly restored after a 5-minute washout period, without differences among diet groups (Figure 4).

Compared to baseline conditions, sevoflurane decreased the microvascular filling velocity (β) and tended to increase

Table 1: Characteristics after 8 weeks of diet feeding.

	Control diet	Western diet
Caloric intake (kcal/100 gBW)	124 ± 6	129 ± 7
Blood/plasma characteristics (n = 8)		
6 h fasting plasma glucose (mmol/L)	8.6 ± 0.7	10.7 ± 1.1*
6 h fasting plasma insulin (pmol/L)	933 ± 383	1524 ± 353*
6 h fasting plasma free fatty acids (mmol/L)	0.26 ± 0.10	0.46 ± 0.24*
6 h fasting plasma triglycerides (mmol/L)	0.68 ± 0.18	3.33 ± 1.19*
6 h fasting plasma HDL cholesterol (mg/dL)	112.3 ± 11.8	61.9 ± 6.7*
6 h fasting plasma LDL/VLDL cholesterol (mg/dL)	15.6 ± 2.2	27.3 ± 7.0*
Hematocrit (%)	50.7 ± 3.0	47.9 ± 2.2*
Body composition (n = 8)		
Body weight (g)	410 ± 27	478 ± 25*
Heart weight (g)	1.19 ± 0.06	1.34 ± 0.15*
Liver weight (g)	10.7 ± 1.0	14.2 ± 1.0*
Epididymal fat weight (g)	6.0 ± 0.8	11.4 ± 2.1*
Perirenal fat weight (g)	8.0 ± 1.5	16.7 ± 3.8*
Tibia length (mm)	42.0 ± 0.5	41.8 ± 1.0
Hemodynamics (n = 18)		
Heart rate (bpm)	405 ± 36	393 ± 48
Systolic blood pressure (mmHg)	145 ± 26	144 ± 31
Diastolic blood pressure (mmHg)	117 ± 21	116 ± 26
Mean arterial pressure (mmHg)	126 ± 22	125 ± 27

Data are mean ± SD, n = 8–18; * $p < 0.05$ versus control diet.

Table 2: Myocardial dimensions after 8 weeks of diet feeding during baseline and after sevoflurane exposure.

	Baseline		Sevoflurane	
	Control diet	Western diet	Control diet	Western diet
Diastolic lumen diameter (mm)	5.7 ± 0.6	5.5 ± 0.8	5.4 ± 0.8	5.5 ± 0.8
Systolic lumen diameter (mm)	2.0 ± 0.4	2.7 ± 0.7*	2.3 ± 0.5	3.4 ± 0.6*#
Diastolic wall thickness (mm)	1.8 ± 0.1	1.9 ± 0.2*	1.6 ± 0.2	1.9 ± 0.2*
Systolic wall thickness (mm)	3.3 ± 0.3	3.1 ± 0.4	3.0 ± 0.4	2.9 ± 0.2

Data are mean ± SD, n = 9–18; two-way ANOVA with Bonferroni post hoc analyses, * $p < 0.05$ diet effect, # $p < 0.05$ sevoflurane effect.

microvascular blood volume (A) in controls, while this observation was absent in western diet-fed animals. Overall, this resulted in an unchanged estimate of perfusion in both diet groups (Figure 2).

Sevoflurane additionally increased end-systolic lumen diameter in western diet-fed rats compared to baseline conditions, which resulted in further impaired systolic function in western diet-fed rats compared to control rats (Figure 3).

4. Discussion

In the present study, we examined the effect of sevoflurane anesthesia on myocardial perfusion and systolic function in western diet-fed rats. We found that short-term western diet feeding resulted in a mild type 2 diabetic phenotype (prediabetes), which was associated with impaired myocardial perfusion and systolic dysfunction. Sevoflurane had no additional effect on myocardial perfusion in healthy and prediabetic rats, while it impaired systolic function in healthy rats and even further impaired systolic function in prediabetic rats. These results suggest that sevoflurane leads to uncoupling of myocardial perfusion and function, irrespective of the metabolic state.

An interesting finding in this study is that myocardial perfusion and function were both decreased in diabetic rats compared to healthy controls. Previously, we found that myocardial perfusion and function were unaffected in high fat diet-induced glucose intolerant rats compared to healthy controls [14]. In the present study rats were exposed to a more severe western diet, which resulted in more pronounced disturbances in the cardiometabolic condition of the rats. Moreover, it was previously shown that the degree of reduction in myocardial blood flow reserve during acute hyperglycemia correlated to the severity of insulin resistance [24].

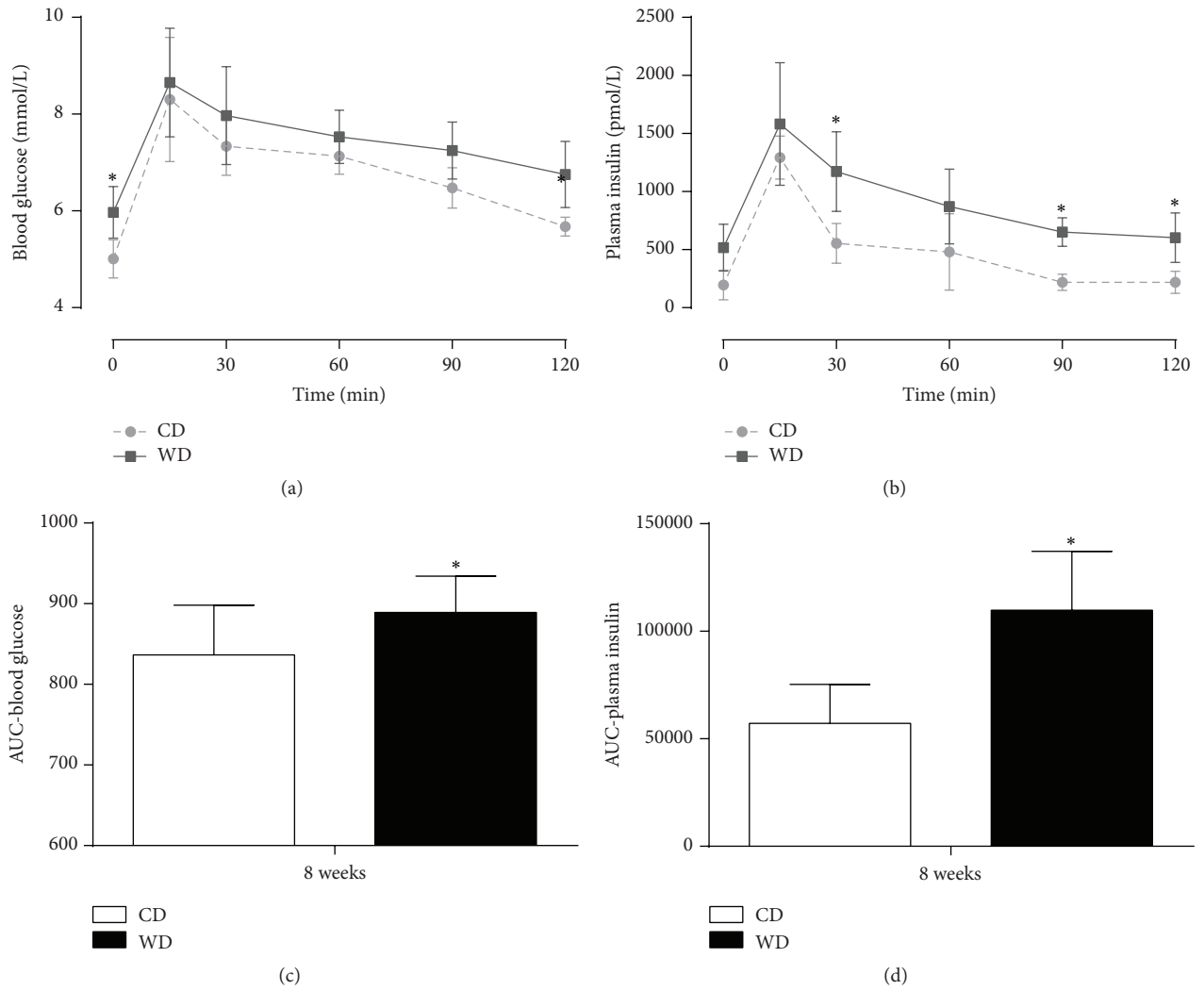

Figure 1: Oral glucose tolerance after 8 weeks of diet feeding. Blood glucose (a), plasma insulin (b), and area under the curve (AUC; (c) and (d)) during an oral glucose tolerance test in rats after 8 weeks of control diet (CD) and western diet (WD) feeding. Data are mean ± SD, $n = 6$, t-test, one- and two-way ANOVA with repeated measures, and Bonferroni post hoc analyses, $^{*}p < 0.05$ versus CD.

Taken together, these results suggest that impairment of myocardial perfusion is related to the severity of the diabetic state.

During surgery, the balance between myocardial energy supply and demand is challenged by extrinsic factors such as anesthetics. The volatile anesthetic sevoflurane exerts contrasting effects on myocardial perfusion in healthy animals and subjects. Previously it has been shown that sevoflurane did not alter myocardial blood flow in healthy rats [25] and cardiovascular healthy patients [6] compared to the awake condition. In contrast, others described decreased myocardial blood flow in healthy rats under general anesthesia with α-chloralose [8], dogs anesthetized with pentobarbital and fentanyl [9], and awake pigs [10]. The present study showed that sevoflurane did not affect myocardial perfusion, despite decreased arterial blood pressure, heart frequency, and rate pressure product in healthy rats. While myocardial perfusion remained unchanged, sevoflurane decreased microvascular filling velocity (β) and increased microvascular blood volume (A). The microvascular filling velocity is a parameter of the capillary exchange rate providing an estimate of the speed of erythrocytes through the capillaries, while microvascular blood volume suggests the surface area for exchange of nutrients and correlates with oxygen consumption. Our observations are in contrast with a previously performed study in cardiovascular healthy subjects by our group, where we showed that sevoflurane decreased myocardial blood volume and increased the microvascular filling velocity [6]. A possible mechanism to explain these differences may be derived by the differences in heart rate among species. We found a decrease in heart rate, while in healthy subjects an increase in heart rate was shown [6]. However, also decreased [8] or unchanged [25] heart rate in healthy rats is found during sevoflurane exposure. In addition to species variation and the use of different experimental techniques [23], administration of general anesthetics may explain the contrasting observations, as this may distinctly alter hemodynamics compared to the awake state. In the present study all rats were

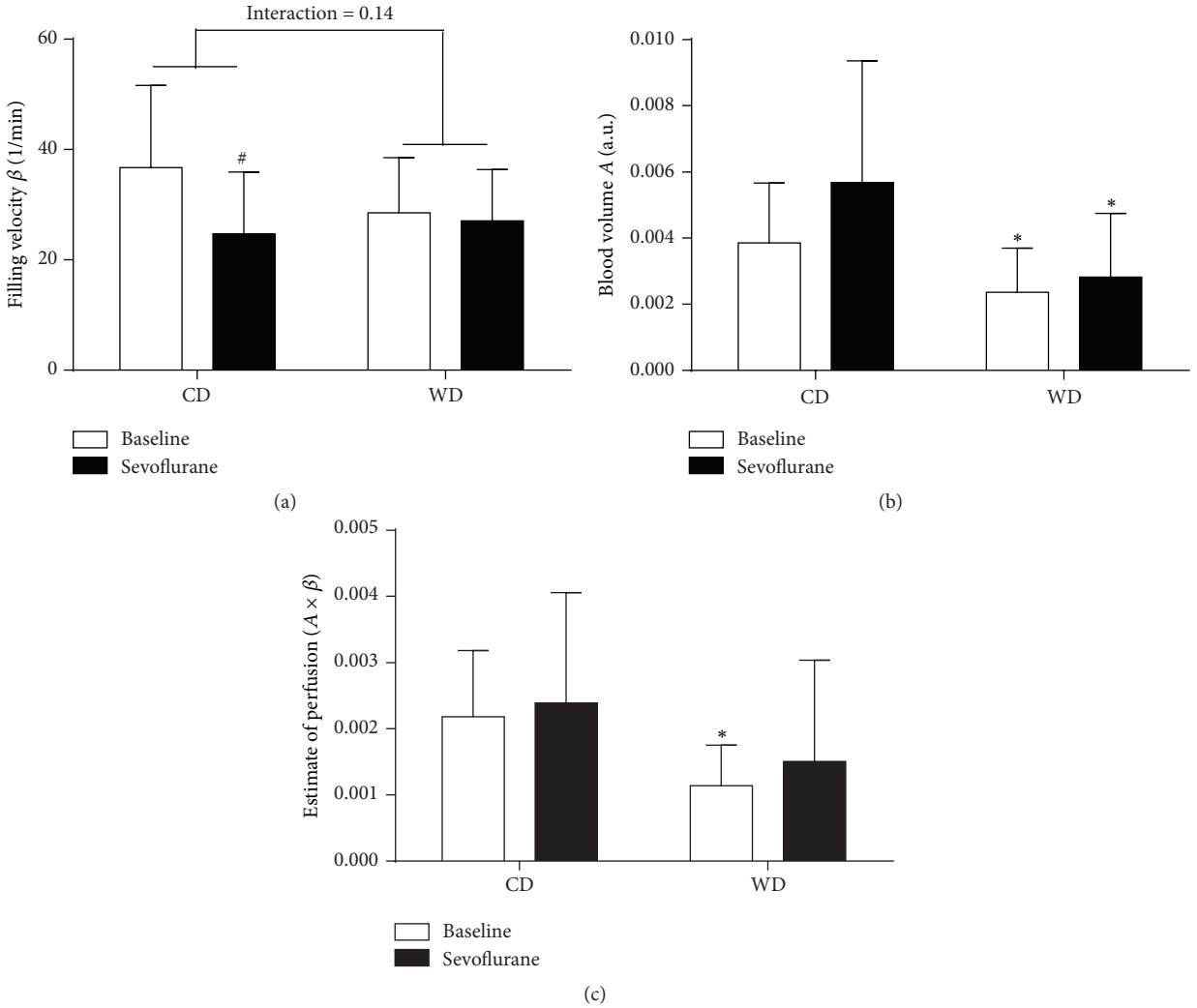

Figure 2: Effect of sevoflurane on myocardial perfusion in prediabetic rats. Microvascular blood volume A (a), microvascular filling velocity β (b), and estimate of perfusion (c) measured with contrast echocardiography in rats fed a control diet (CD) or western diet (WD) for 8 weeks during baseline conditions and after 5 minutes of sevoflurane exposure. Data are expressed as mean ± SD, n = 9–13; two-way ANOVA with Bonferroni post hoc analyses, $^{*}p < 0.05$ diet effect, $^{\#}p < 0.05$ sevoflurane effect.

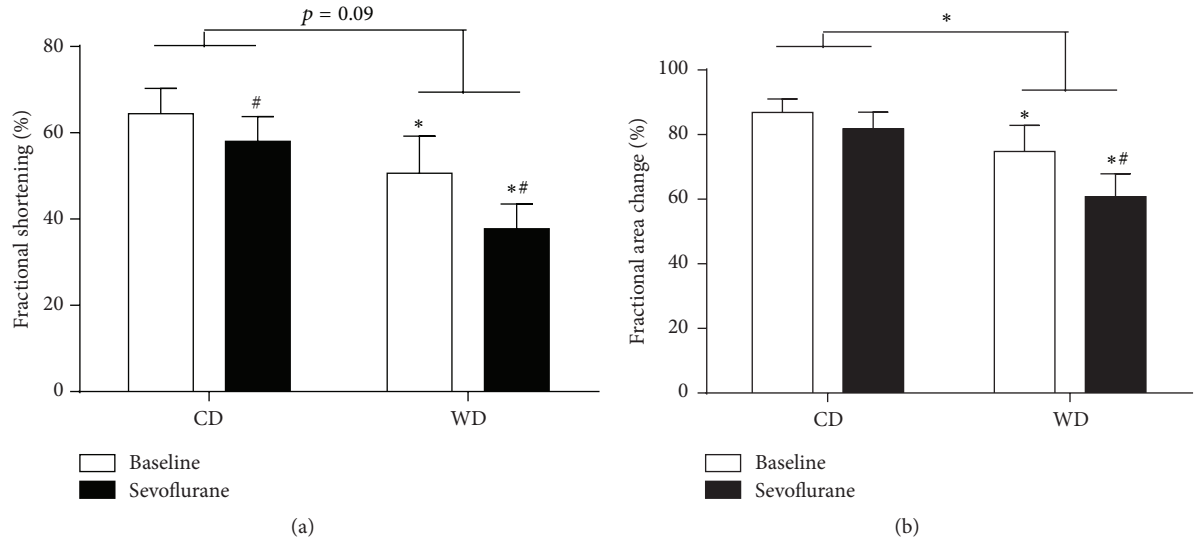

Figure 3: Effect of sevoflurane on systolic function in prediabetic rats. Systolic function, as represented by the fractional shortening (a) and fractional area change (b), measured with echocardiography in rats fed a control diet (CD) or western diet (WD) for 8 weeks during baseline conditions and after 5 minutes of sevoflurane exposure. Data are expressed as mean ± SD, n = 9–18; two-way ANOVA with Bonferroni post hoc analyses, $^{*}p < 0.05$ diet effect, $^{\#}p < 0.05$ sevoflurane effect.

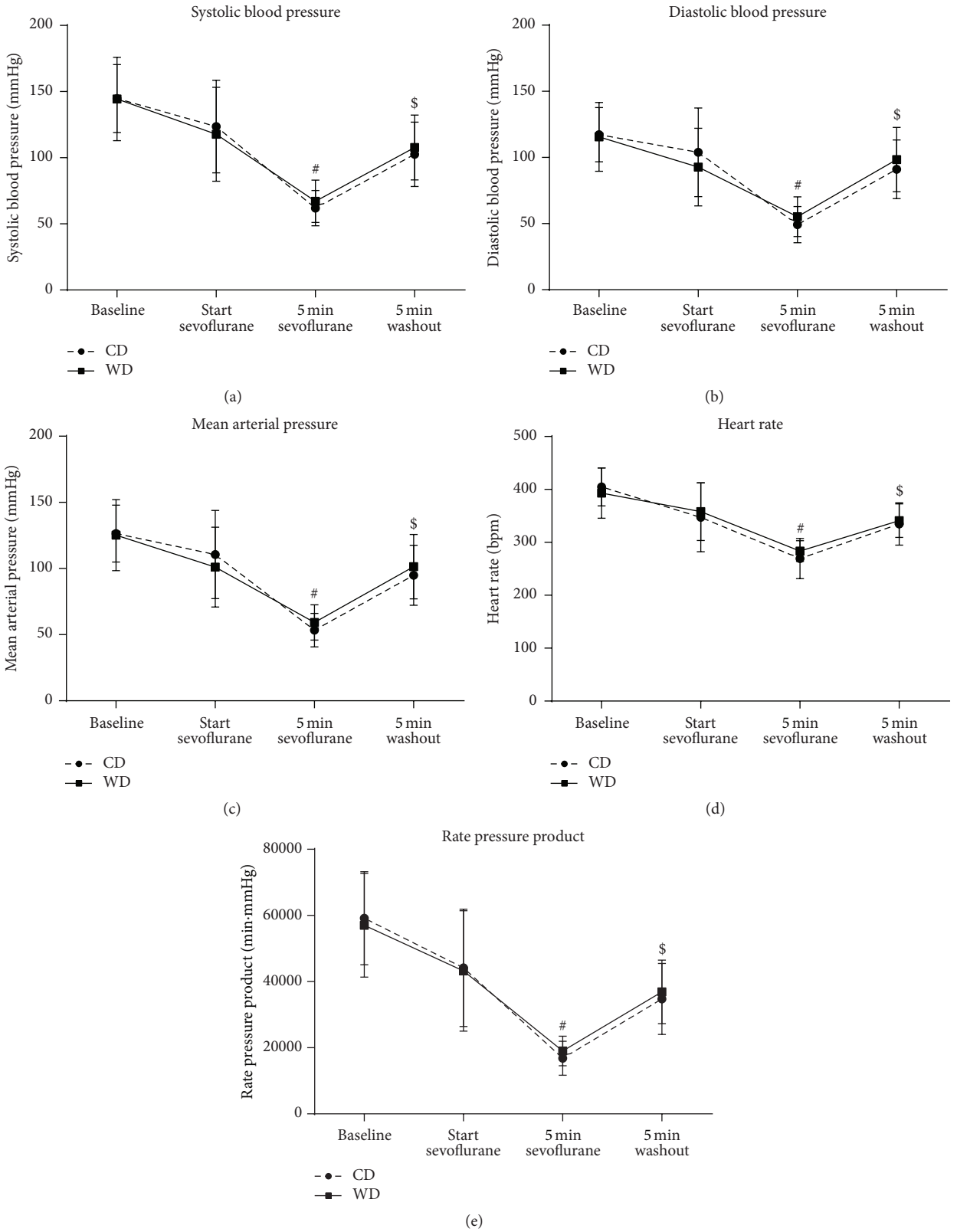

Figure 4: Hemodynamics during sevoflurane exposure. Systolic blood pressure (a), diastolic blood pressure (b), mean arterial pressure (c), heart rate (d), and rate pressure product (e) during baseline conditions, before sevoflurane exposure, after 5 minutes of sevoflurane and after 5 minutes of washout period in rats fed a control diet (CD) or western diet (WD) for 8 weeks. Data are mean ± SD, $n = 16$–18, two-way ANOVA with repeated measurements, and Bonferroni post hoc analyses, $^{\#}p < 0.05$ sevoflurane effect, $^{\$}p < 0.05$ washout effect.

primarily sedated with S-ketamine and diazepam, because it is not feasible to perform contrast echocardiography in awake rats. As S-ketamine and diazepam also have intrinsic cardiodepressive effects [26, 27], this might have blurred the direct effect of sevoflurane on myocardial function. However, despite the use of several anesthetics, myocardial function was only slightly affected, whereas myocardial perfusion remained unaffected. Taken together, although sevoflurane anesthesia slightly impaired myocardial function, myocardial perfusion was not affected in healthy rats.

Preservation of myocardial perfusion during surgery is particularly important in patients with increased risk for perioperative cardiac complications, such as diabetes. Recently, our group showed that sevoflurane decreased myocardial perfusion in type 2 diabetic patients compared to healthy controls. Moreover, we observed a trend towards a lower endothelium-independent vasodilation capacity in type 2 diabetic patients under sevoflurane anesthesia, while endothelium-dependent vasodilation was not affected [19]. Our present results show that sevoflurane has a stronger cardiodepressive effect in prediabetic rats, whereas myocardial perfusion remained unaffected. Interestingly, systolic function was partly restored when sevoflurane was withdrawn (unpublished data). Under physiological conditions, myocardial blood flow and function are in balance [28]. In our prediabetic rats, myocardial perfusion and myocardial function were decreased. However, during sevoflurane anesthesia, myocardial perfusion was maintained, while myocardial function was further decreased in prediabetic rats. This uncoupling of perfusion and function suggests that, despite increased microvascular blood volume and decreased microvascular filling velocity, myocardial function cannot be maintained. Moreover, It should be kept in mind that the effects of sevoflurane are studied on top of S-Ketamine and diazepam anesthesia. However, the cardiodepressive effects of these agents do not explain our findings.

5. Conclusions

In conclusion, sevoflurane anesthesia maintained myocardial perfusion, while it impaired systolic function in healthy rats and even further impaired systolic function in prediabetic rats. Our findings suggest that sevoflurane anesthesia uncouples myocardial function and myocardial perfusion, irrespective of the metabolic state. This uncoupling might increase the vulnerability of the heart for an oxygen supply and demand mismatch and consequent ischemia during surgery.

Conflict of Interests

No conflict of interests, financial or otherwise, is declared by the authors.

Acknowledgments

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Research Article

Expression of the Receptor for Advanced Glycation End Products in Epicardial Fat: Link with Tissue Thickness and Local Insulin Resistance in Coronary Artery Disease

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Increased expression of receptor for advanced glycation end products (RAGE) in adipose tissue has been associated with inflammation, adipocyte hypertrophy, and impaired insulin signal. Epicardial adipose tissue (EAT), a visceral fat surrounding the myocardium, is potentially involved in the onset/progression of coronary artery disease (CAD). To date, the role of RAGE in EAT has not been explored much. We examined whether the RAGE expression in EAT was associated with EAT adiposity and metabolic dysfunctions normally found in CAD patients. EAT samples were obtained from 33 patients undergoing open-heart surgery. EAT expression of RAGE, GLUT4, adiponenctin, GLO1, HMGB1, TLR-4, and MyD88 was analyzed by microarray. EAT thickness was quantified by echocardiography. Anthropometric measures and clinical parameters were taken. BMI, HOMA-IR, and LAP indices were calculated. With increasing RAGE expression in EAT we observed increases in EAT thickness, reduced expression of GLUT4, adiponectin, and GLO1, and elevations of HMGB1, TLR-4, and MyD88. There were significant correlations between RAGE and EAT thickness and between RAGE and the genes. LAP was higher in patients with increased RAGE expression. Our data suggest that in CAD patients RAGE may be involved in promoting EAT adiposity and metabolic dysfunction, such as impaired insulin signaling.

1. Introduction

The role of epicardial adipose tissue (EAT) in the onset and progression of coronary artery disease (CAD) is recognized [1], but the mechanisms and mediators promoting and linking EAT dysfunctions and CAD still need to be understood and described better.

The receptor for advanced glycation end products (RAGE) is a multiligand receptor that binds advanced glycation end products (AGE) and other endogenous nonglycated peptides, such as ligand mobility group box 1 (HMGB1), many of which are important regulators of the inflammatory process [2]. Although RAGE was initially implicated in cardiovascular complications related to diabetes [3, 4], recent reports have suggested its central role in inflammation and inflammation-associated dysfunctions, such as obesity, metabolic syndrome, and atherosclerosis, even in nondiabetic conditions [5–8].

In CAD, EAT displays inflammatory features due to infiltrated macrophages and T cells and reduced production of protective factors in favor of detrimental proinflammatory

mediators. This inflammatory condition may in turn contribute to the progression of atherosclerosis besides exacerbating metabolic complications in EAT [9, 10].

On the basis of the potential role of RAGE in adipogenesis, inflammation, and insulin resistance [5–8], we explored whether its expression in EAT was associated with EAT adiposity and the metabolic dysfunctions, such as impaired insulin signaling, normally found in CAD patients.

2. Materials and Methods

2.1. Study Population. Thirty-three male CAD patients undergoing coronary artery bypass grafting (CABG) surgery were enrolled in the study during their hospitalization. Exclusion criteria were acute myocardial infarction within the last month, previous or current malignant disease, major abdominal surgery within the previous 6 months, renal and liver diseases, end-stage heart failure, and more than 3% change in body weight in the previous three months. Anthropometric measures were recorded. Body mass index (BMI) was calculated by dividing the weight (in Kg) by the square of the height (in meters). The study protocol, conducted in accordance with the Declaration of Helsinki, as revised in 2013, was approved by the local ethics committee (ASL Milano Due, Protocol 2516). Patients gave their written informed consent to the protocol.

2.2. Blood Collection. Blood samples were collected after overnight fasting into pyrogen-free tubes with ethylenediaminetetraacetic acid as anticoagulant. Fasting glucose, glycated hemoglobin, insulin, total and HDL cholesterol, triglycerides, and C-reactive protein (CRP) were quantified with commercial kits using Cobas 6000 analyzer (Roche Diagnostics, Milan, Italy), as previously reported [11, 12]. Precision was determined by the manufacturer using human samples and controls in an internal protocol with repeatability ($n = 21$) and intermediate precision (3 aliquots per run, 1 run per day, 21 days). Results for repeatability were glucose, 1% at 98.8 mg/dL and 0.9% at 245 mg/dL; HbA1c, 1.3% at 5.3% and 1.1% at 9.9%; insulin, 1.9% at 6.36 μU/mL and 1.9% at 20.9 μU/mL; total cholesterol, 1.1% at 88.5 mg/dL and 0.9% at 183 mg/dL; HDL, 0.4% at 53.4 mg/dL and 1% at 34.4 mg/dL; triglycerides, 0.9% at 125 mg/dL and 0.8% at 212 mg/dL; and CRP, 1.2% at 3.35 mg/L and 1.3% at 44.4 mg/L.

Results for intermediate precision were glucose, 1.3% at 96.9 mg/dL and 1.1% at 241 mg/dL; HbA1c, 1.4% at 5.3% and 1.5% at 9.9%; insulin, 2.6% at 6.36 μU/mL and 2.8% at 20.9 μU/mL; total cholesterol, 1.6% at 89.3 mg/dL and 1.6% at 188 mg/dL; HDL, 0.9% at 51.8 mg/dL and 1.5% at 34 mg/dL; triglycerides, 2% at 123 mg/dL and 1.6% at 206 mg/dL; and CRP, 2.9% at 29.1 mg/L and 1.9% at 43.6 mg/L.

LDL cholesterol was calculated with the Friedewald formula. Insulin resistance index (HOMA-IR) was calculated as follows: HOMA-IR = fasting insulin [μU/mL] × fasting glucose [mmol/L]/22.5. The formula used for the lipid accumulation product (LAP) was (waist circumference [WC, cm] − 65) × (triglycerides [TG, mmol/L]).

2.3. Quantification of EAT. EAT quantification by echocardiography was performed in addition to the routine clinical examinations just before CABG surgery, usually one or two days before. Patients were examined by echocardiography using an M-mode color-Doppler VSF (Vingmed-System Five; General Electric, Horten, Norway) with a 2.5–3.5 MHz transducer probe. EAT thickness was measured as previously reported [13].

2.4. EAT Collection. EAT biopsy samples were harvested adjacent to the proximal right coronary artery prior to starting cardiopulmonary bypass pumping. Samples were stored in Allprotect Tissue Reagent (Qiagen, Hilden, Germany) at −20°C until RNA extraction.

2.5. RNA Extraction and Gene Expression Analysis. Total RNA was extracted from tissue with the RNeasy Lipid Tissue Kit according to the manufacturer's procedures (Qiagen). RNA concentration was quantified by NanoDrop 2000 (Thermo Scientific, Wilmington, Germany) and RNA integrity was assessed using the Agilent RNA 6000 Nano Kit and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Gene expression was analyzed with a one-color microarray platform (Agilent): 50 ng of total RNA was labeled with Cy3 using the Agilent Low Input Quick-Amp Labeling Kit-1 color, according to the manufacturer's directions. cRNA was purified with the RNeasy Mini Kit (Qiagen) and the amount and labeling efficiency were measured with NanoDrop. Hybridization was done using the Agilent Gene Expression Hybridization Kit and scanning with the Agilent G2565CA Microarray Scanner System.

Data were processed using Agilent Feature Extraction Software (10.7) with the single-color gene expression protocol and raw data were analyzed with ChipInspector Software (Genomatix, Munich, Germany). In brief, raw data were normalized on a single-probe level based on the array mean intensities and statistics were calculated using the SAM algorithm by Tusher et al. [14]. Changes were determined from normalized data.

2.6. Statistical Analysis. Data are expressed as mean ± SD or number and percentage. The normality of data distribution was assessed by the Kolmogorov-Smirnoff test. Quantitative variables were compared using Student's unpaired t-test and Mann-Whitney and Kruskal-Wallis tests, as appropriate. The χ^2 test was used for categorical variables. Relations between parameters were examined by the Spearman correlation test. Data were analyzed using GraphPad Prism 5.0 biochemical statistical package (GraphPad Software, San Diego, CA). A p value < 0.05 was considered significant.

3. Results

3.1. Patients. Demographic, anthropometric, and clinical characteristics of the patients are shown in Table 1. Patients were classified into two groups (Q1 and Q2) according to the median value of RAGE expression in EAT (168.33 arbitrary

Table 1: Demographic, anthropometric, and biochemical characteristics of coronary artery disease patients included in the study before and after classification according to the median value of RAGE expression at EAT level (Q1 and Q2 groups).

	Median	25th–75th percentiles	Range	Q1 (mean ± SD)	Q2 (mean ± SD)	p
Age (years)	68.00	57.50–71.70	50.00–86.00	68.18 ± 2.99	66.19 ± 2.09	0.67
Weight (Kg)	75.00	65.75–81.50	52.00–135.00	72.88 ± 2.84	84.17 ± 6.12	0.33
BMI (kg/m^2)	26.20	23.76–27.90	19.12–41.80	25.14 ± 0.71	28.62 ± 1.44	0.08
Waist (cm)	102.00	93.50–109.05	72.00–144.00	95.94 ± 3.25	109.60 ± 3.61	<**0.01**
EAT thickness (mm)	7.50	5.78–8.00	3.00–10.00	5.50 ± 0.67	7.58 ± 0.44	<**0.05**
Fasting glucose (mg/dl)	82.50	77.50–104.50	64.00–177.00	99.24 ± 7.83	91.53 ± 6.59	0.79
Fasting insulin (μU/ml)	7.11	4.17–10.93	3.19–45.06	6.97 ± 0.71	9.07 ± 1.90	0.53
HbA1C (%)	4.68	3.53–5.52	2.79–7.10	4.76 ± 0.36	4.58 ± 0.30	0.71
Total cholesterol (mg/dl)	153.00	138.00–180.30	88.00–261.00	169.80 ± 10.20	146.40 ± 6.81	0.08
HDL cholesterol (mg/dl)	44.00	34.25–49.25	23.00–69.00	44.13 ± 2.50	41.73 ± 3.53	0.58
LDL cholesterol (mg/dl)	83.60	71.50–109.30	17.60–192.00	104.40 ± 9.89	82.57 ± 4.45	0.07
Triglycerides (mg/dl)	107.00	88.50–143.00	64.00–244.00	114.60 ± 9.43	130.10 ± 15.57	0.40
CRP (mg/dl)	0.20	0.10–0.95	0.00–7.90	0.76 ± 0.37	1.23 ± 0.54	0.44
Systolic blood pressure (mmHg)	130.00	120.00–140.00	110.00–150.00	129.10 ± 2.51	131.30 ± 3.26	0.61
Diastolic blood pressure (mmHg)	70.00	70.00–80.00	60.00–80.00	74.55 ± 1.58	83.00 ± 1.49	0.10
	n	%		Q1 (n, %)	Q2 (n, %)	p
Smokers	14	42.42		6, 42.85	8, 57.15	0.73
Diabetes mellitus	9	27.27		4, 44.44	5, 55.56	0.70
Hypertension	24	72.73		11, 45.83	13, 54.17	0.44
Dyslipidemia	20	60.61		7, 35.00	13, 65.00	<**0.05**
Aspirin	19	57.58		6, 31.58	13, 68.42	<**0.05**
Antidiabetics	6	18.18		3, 50.00	3, 50.00	1
ACEI/ARB	24	72.73		10, 41.67	14, 58.33	0.12
β-Blockers	16	48.48		7, 43.75	9, 56.25	0.49
Calcium channel blockers	6	18.18		3, 50.00	3, 50.00	1
Statins	22	66.67		8, 36.36	14, 63.64	<**0.05**

ACEI: angiotensinogen-converting enzyme inhibitor; ARB: angiotensin receptor blockade; BMI: body mass index; CRP: C-reactive protein; EAT: epicardial adipose tissue; HbA1C: glycated hemoglobin. Data are expressed as median, 25th–75th percentiles and range or number (n), and % or mean ± standard deviation (SD).

unit, A.U.). Patients were 17 in Q1 and 16 in Q2 and the mean value of RAGE expression was 140.29 A.U. in Q1 and 233.33 A.U. in Q2 ($p < 0.001$). No statistically significant differences were observed in clinical parameters between the two groups. There were higher percentages of dyslipidemic patients and more patients taking statin and aspirin in the Q2 group ($p < 0.05$ for all).

3.2. Increased RAGE Expression in EAT Is Associated with Greater Echocardiographic EAT Thickness and Higher Adiposity Indices. Echocardiographic EAT thickness and WC, a marker of visceral fat distribution, were higher in group Q2 than Q1 ($p < 0.05$ and $p < 0.01$, resp.) (Figure 1(a) and Table 1). Positive correlations were seen between RAGE-EAT thickness ($r = 0.48$, $p < 0.05$) and RAGE-WC ($r = 0.35$, $p < 0.05$) (Figure 1(b)). The relation between increased RAGE expression and greater EAT thickness was confirmed by classifying patients according to the median EAT thickness (7.5 mm). RAGE expression in the upper group (EAT thickness > 7.5 mm) was about 1.3 times higher ($p < 0.05$) than in the lower group (EAT thickness < 7.5 mm) (Figure 1(c)).

3.3. Increased RAGE Expression in EAT Is Associated with Lower GLUT4 and Adiponectin Expression and a Higher LAP Index. Patients in the upper group of RAGE expression in EAT (Q2) had about 1.6 times lower levels of both the insulin-sensitizing adipokine adiponectin and the insulin-responsive glucose transporter 4 (GLUT4) than in the Q1 group ($p < 0.05$ for both) (Figure 2). There was an inverse correlation between RAGE-adiponectin ($r = -0.40$, $p < 0.05$) and RAGE-GLUT4 ($r = -0.60$, $p < 0.001$).

Examining the relation between RAGE expression in EAT and LAP and HOMA-IR, two parameters of insulin resistance, only LAP was higher in Q2 than in Q1 (Figure 2).

3.4. Increased RAGE Expression in EAT Is Associated with Lower Expression of the Antioxidant Glyoxalase 1 System and Higher HMGB1, TLR-4, and MyD88. Patients in the Q2 group of RAGE expression in EAT had about a 1.3 times lower level of glyoxalase 1 (GLO1), a system playing a critical role in the prevention of glycation reactions mediated by methylglyoxal, glyoxal, and other RAGE ligands, and about 1.2 times the level of the endogenous RAGE ligand HMGB1 compared to group Q1 ($p < 0.01$ for both) (Figure 3(a)). Both TLR-4 and MyD88,

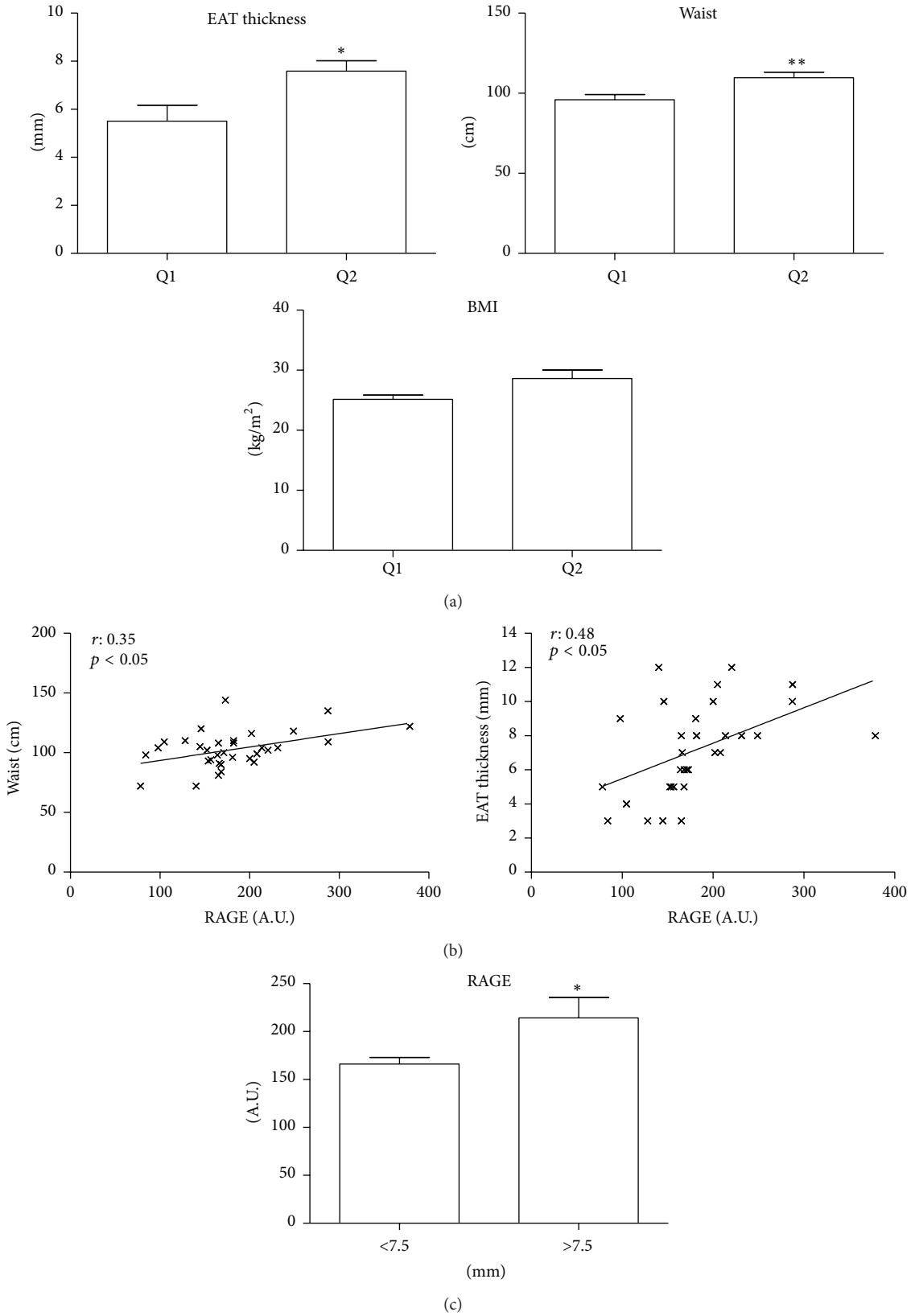

Figure 1: Relationship between RAGE expression in EAT, EAT thickness, and anthropometric indices in CAD patients. (a) CAD patients were stratified into two groups (Q1 and Q2) on the basis of the median RAGE expression in EAT, and EAT thickness, waist circumference, and BMI were compared in the two groups. (b) Spearman correlation analysis between mRNA RAGE level in EAT and waist circumference and EAT thickness. (c) CAD patients were stratified into two groups (Q1 and Q2) on the basis of the median EAT thickness (7.5 mm) and the levels of RAGE expression were compared in the two groups. A.U.: arbitrary unit. Data are expressed as mean ± SD; $^{*}p < 0.05$, $^{**}p < 0.01$.

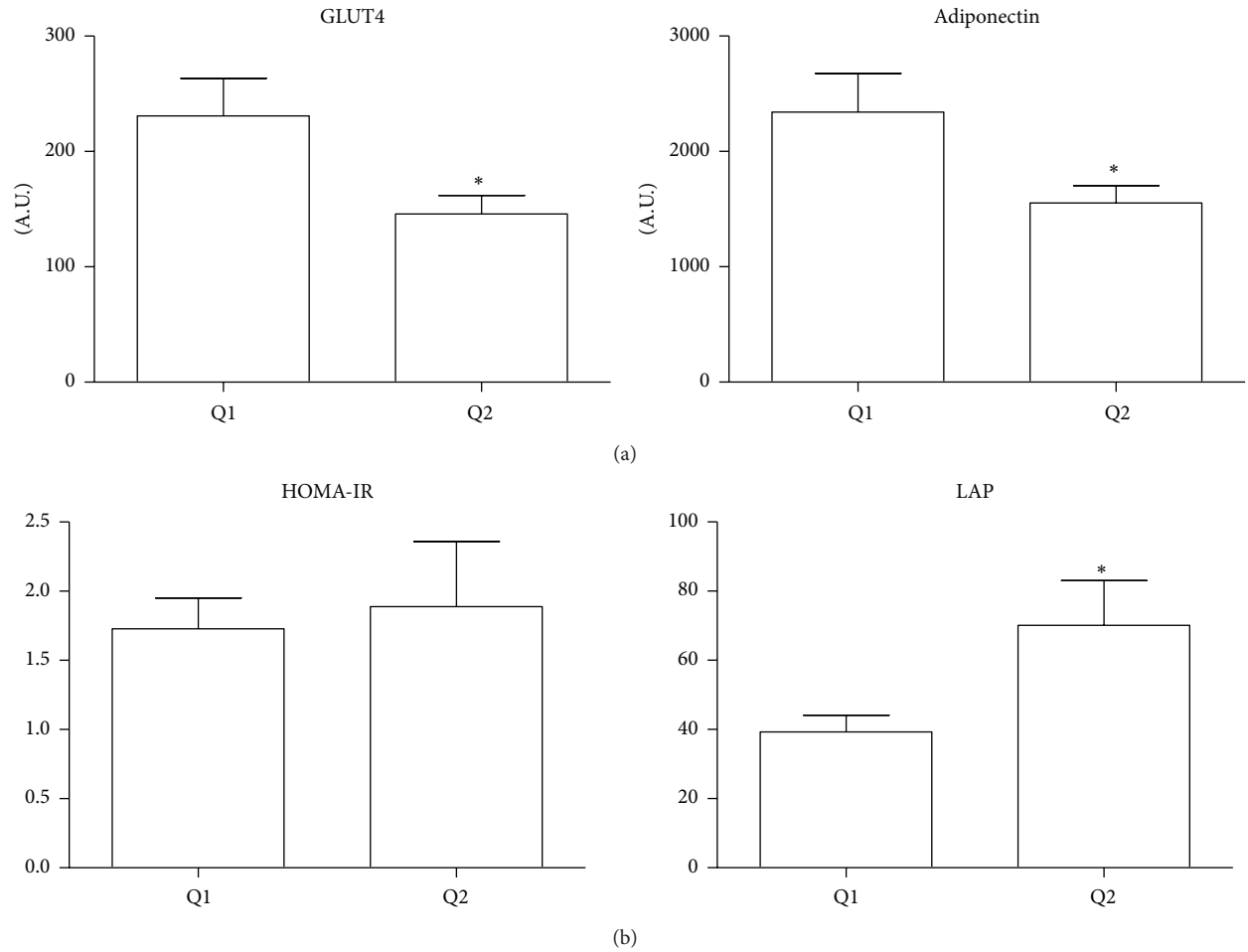

Figure 2: Relationship between RAGE expression in EAT and indices of insulin sensitivity. CAD patients were stratified into two groups (Q1 and Q2) on the basis of the median RAGE expression in EAT. (a) Gene expression of GLUT4 and adiponectin in EAT was compared in the two groups. (b) HOMA-IR and LAP were compared in the two groups. Data are expressed as mean ± SD; $^{*}p < 0.05$.

important in the activation of the innate immune system, were higher in group Q2 (about 1.4 times, $p < 0.05$ for both) (Figure 3(b)).

Correlation analyses indicated an inverse correlation between RAGE and GLO1 ($r = -0.65$, $p < 0.0001$) and a positive association between RAGE-TLR-4 ($r = 0.51$, $p < 0.001$) and RAGE-MyD88 ($r = 0.48$, $p < 0.01$).

4. Discussion

To the best of our knowledge this is the first human study exploring the existence of an association between the expression of RAGE in EAT, EAT metabolic dysfunctions, and adiposity in CAD patients. The findings indicate that EAT thickness as well as local tissue inflammation and insulin sensitivity seems related to local expression of RAGE.

Previous *in vitro* and animal studies suggested that RAGE could be involved in the progression of obesity, with a direct role in promoting adipocyte hypertrophy [7]. RAGE −/− mice at 20 weeks of age had lower weight and lower epididymal adipose tissue weight and adipocyte size than wild type mice [7]. The observation that adenoviral RAGE overexpression in 3T3-L1 adipocytes markedly induced a hypertrophic phenotype, which was suppressed by RAGE silencing, also supports a role for RAGE in promoting adipocyte hypertrophy [7].

Our data seem to confirm that RAGE is involved in adiposity, mainly visceral, in humans too. In fact, with increasing local expression of RAGE, we observed increases in the thickness of EAT, an acknowledged visceral fat, and in WC, which serves as a marker of visceral fat accumulation. Whether this RAGE-related EAT expansion involved adipocyte hypertrophy needs to be explored further. In fact, we noted a hypertrophic state associated with the increased RAGE expression (data not shown), but since the number of patients with enough tissue for this analysis was limited, these results can only be considered preliminary.

In this study we did not directly explore the mechanisms promoting RAGE upregulation, but on the basis of previous data also from our group it would appear that both the increased inflammatory state described in EAT in CAD patients and the greater local accumulation of AGE products in expanding adipose tissue may promote this [9, 12, 15–17].

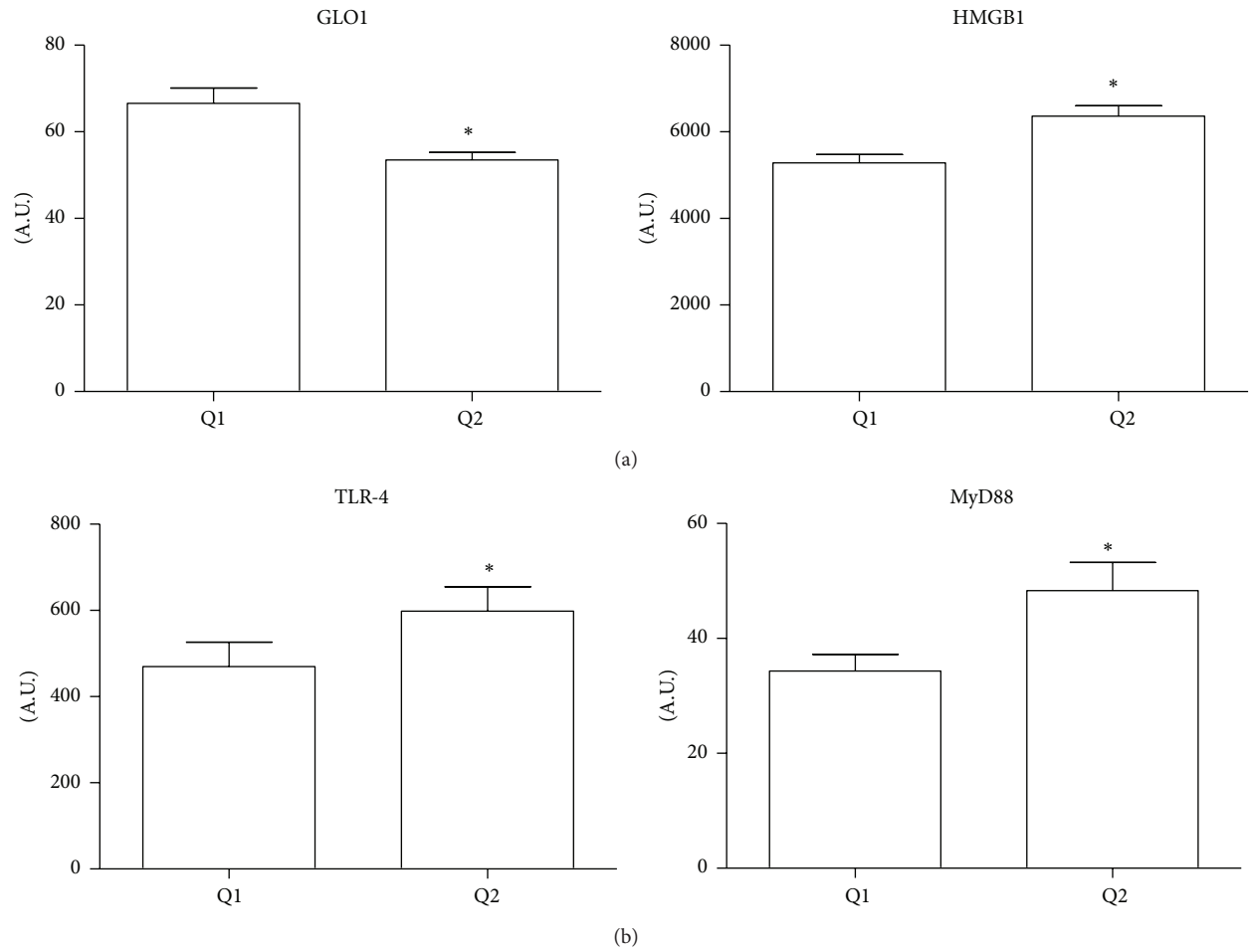

Figure 3: Relationship between RAGE expression in EAT and EAT inflammation. CAD patients were stratified into two groups (Q1 and Q2) on the basis of the median RAGE expression in EAT. (a) Gene expression of GLO1 and HMGB1 in EAT was compared in the two groups; (b) TLR-4 and MyD88 levels were compared in the two groups. A.U.: arbitrary unit. Data are expressed as mean ± SD; $^{*}P < 0.05$.

The increase in the local production of damaging agents and reduced protection against them is also borne out by the marked reduction in GLO1, the major detoxification enzyme that protects against AGE [18], and the higher expression of HMGB1, an endogenous mediator of inflammation able to bind RAGE, promoting its expression and amplifying the inflammatory response also through activation of toll-like receptor (TLR)/MyD88 pathways. It has recently been suggested that RAGE not only shares several common ligands with the TLRs, such as HMGB1, but may also interact with MyD88, an important intracellular adaptor protein used by these receptors [19]. There is increasing evidence, therefore, of their potential synergism in amplifying inflammatory responses and our findings too suggest a link between TLR-4/MyD88 and RAGE hyperexpression.

Our data also confirm that some important metabolic dysfunctions of EAT in patients with CAD may be related to RAGE overexpression. Monden et al. [7] indicated that RAGE overexpression reduced the genes involved in insulin sensitivity, such as GLUT4 and adiponectin, and attenuated insulin function. We too saw lower levels of both GLUT4 and adiponectin, with increased RAGE expression in EAT. This suggests a potential impairment of local insulin signaling. We examined insulin sensitivity in our CAD patients using the HOMA-IR, a marker of insulin resistance mainly in the liver, and LAP, a continuous variable based on WC and triglyceride concentration, two parameters which reflect tissue lipid accumulation and denote visceral adiposity [20, 21]. Our observation that RAGE expression in EAT was mainly related to LAP reinforced the idea of a strong correlation between local RAGE overexpression, fat accumulation, and impaired insulin sensitivity.

Our study has some limitations. The first one is the lack of data on protein expression. Since isolation of EAT during surgery is a delicate and difficult procedure and the amount of tissue isolated is often poor and not enough to perform both gene and protein expression analyses, in this study we first decided to carry out a gene expression study. The lack of quantification of local AGE as well as the evaluation of other molecules, which may promote RAGE upregulation, may also represent a second important limit. The third limitation is that we included only males, so presently we cannot check

for possible gender-related differences. Only through new patient enrollment, of both sexes, we will be able to perform protein expression quantification to study which specific pathways/molecules drive RAGE expression in EAT as well as clarify the existence of potential gender-related differences. Finally, the comparison between EAT and other kinds of fat depots, not performed in this study, could be helpful to reinforce our data on the role of RAGE in linking EAT metabolic dysfunction and CAD.

5. Conclusions

In conclusion this study's findings suggest the potential involvement of RAGE in promoting EAT dysfunction in CAD patients. Whether RAGE could also be a potential target to reduce EAT-induced cardiovascular and other complications needs to be explored further.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Authors' Contribution

Elena Dozio, Lorenza Tacchini, and Massimiliano Marco Corsi Romanelli designed the study. Elena Dozio, Elena Vianello, and Silvia Briganti acquired and analyzed data. Elena Dozio wrote the paper. John Lamont, Lorenza Tacchini, Gerd Schmitz, and Massimiliano Marco Corsi Romanelli critically revised the paper.

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Review Article

Subclinical Detection of Diabetic Cardiomyopathy with MicroRNAs: Challenges and Perspectives

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The prevalence of cardiac diabetic diseases has been increased around the world, being the most common cause of death and disability among diabetic patients. In particular, diabetic cardiomyopathy is characterized with a diastolic dysfunction and cardiac remodelling without signs of hypertension and coronary artery diseases. In an early stage, it is an asymptomatic disease; however, clinical studies demonstrate that diabetic myocardia are more vulnerable to injury derived by acute myocardial infarct and are the worst prognosis for rehabilitation. Currently, biochemical and imaging diagnostic methods are unable to detect subclinical manifestation of the disease (prior to diastolic dysfunction). In this review, we elaborately discuss the current scientific evidences to propose circulating microRNAs as promising biomarkers for early detection of diabetic cardiomyopathy and, then, to identify patients at high risk of diabetic cardiomyopathy development. Moreover, here we summarise the research strategies to identify miRNAs as potential biomarkers, present limitations, challenges, and future perspectives.

1. Introduction

The global prevalence of diabetes was estimated as 387 million people in 2014 and expected to increase to 592 million by 2035. Diabetes is a chronic disease that leads to multisystem complications such as nephropathy, retinopathy, neuropathy, and cardiovascular diseases. In 2014 alone about 4.9 million people died due to diabetes-related diseases, cardiovascular disease being the most common cause of death and disability among people with diabetes [1].

Diabetes slowly reduces heart function, (1) promoting the generation of atheroma in coronary arteries (atherosclerosis), reducing oxygen and nutrient supply to cardiac cells [2], (2) impairing autonomic nerve fibres that innervate the blood vessels and heart that produce abnormalities in control heart rate (arrhythmia) and vessels dynamics (cardiac autonomic neuropathy) [3], and (3) reducing contractile capacity of muscle cells and decreasing capillarity irrigation of myocardium (diabetic cardiomyopathy) [4]. While atherosclerosis is well known to increase the risk of heart failure through an episode of ischemia, the weakening of the cardiac fibres by the silent progression of diabetic cardiomyopathy is not accounted due to technical limitations in its subclinical detection. Population-based cohort studies reported by From et al. suggested that patients with heart failure and diabetes but no atherosclerosis had a higher risk of death [5]. In fact, Shah et al. reported that diabetes is related to a higher risk of heart failure hospitalization or death, independently of the left ventricular efficiency fraction levels in patients after a myocardial infarct event; that is, diabetic patients have less capacity to recuperate cardiac functions to normal levels with respect to nondiabetic patients [6].

Diabetic cardiomyopathy was defined first in 1972 as a heart failure without signs of coronary artery disease,

hypertension, or valvular or congenital heart disease by Rubler et al. [7]. In spite of the differences in etiology and metabolic profile, many pathophysiologic features of this cardiomyopathy are shared by diabetes mellitus type 1 (DMT1) and diabetes mellitus type 2 (DMT2) [8].

Considering these common pathophysiological mechanisms, in a prediabetic and diabetic state, plasma level of free fatty acid (FFA) is increased, producing an augmented uptake, accumulation, and oxidation of FFA in the cardiomyocytes. The excess of FFA utilization generates a concomitant suppression of glucose oxidation by the indirect inhibition of the pyruvate dehydrogenase and a downregulation of glucose transporters 1 and 4 (*GLUT1* and *GLUT4)*, establishing a metabolic derangement. The deficiency of glycolysis intermediates decreases the mitochondrial ATP synthesis by oxidative phosphorylation. To compensate this limitation and to attend the high ATP requirement of the heart, β-oxidation of FFA has a prominent role in increasing oxygen consumption and reactive oxygen species (ROS), which upregulates the uncoupling proteins expression to balance the proton transmembrane gradient needed for ATP synthesis [9]. The excessive ROS production stimulates apoptotic signals by ceramide generation and mitochondrial cytochrome c release [10]. Besides the deficiency of energy production, the excitation contraction coupling, essential for cardiac contraction, is altered by impaired intracellular Ca^{+2} handling [9].

The mitochondrial oxidative stress and "glycolated" tissue state produces an endothelial dysfunction and a proinflammatory microenvironment that stimulated the infiltration of macrophages and leukocytes that aggravates heart inflammation and tissue damage [9]. The remodelling of extracellular matrix is characterized by an interstitial and perivascular fibrosis and abnormalities in microvasculature [11]. In a latent subclinical period, metabolic disturbances and structural abnormalities lead to a diastolic dysfunction, which subsequently progresses to left ventricular hypertrophy, contractility reserve impairment, and, eventually, a systolic dysfunction [12].

Currently, therapy for diabetic cardiomyopathy is based on glycaemia control and hypoglycaemic drugs administration and changes in lifestyle (for DMT2), which delay the progression to heart failure but not revert it; however, therapy efficacy can be improved with earliest detection [13]. In this review we discuss the usefulness and limitations of the current methods used in the clinic to diagnose diabetic cardiomyopathy. We summarize the scientific evidences to propose miRNAs as new generation of biomarkers at subclinical stages of this disease by reflexing in biofluids the myocardial metabolic derangement before cardiac dysfunction.

2. Diagnosis of Diabetic Cardiomyopathy

Diabetic cardiomyopathy detection is a challenge in the clinical practice due to lack of any specific pathognomonic histologic changes or imaging characteristics. However, diastolic dysfunction and cardiac hypertrophy (measured by tissue Doppler echocardiography) in the absence of coronary artery disease and hypertension have been considered the two principal hallmarks to propose a diagnosis of diabetic cardiomyopathy in asymptomatic diabetic patients [14, 15].

Imaging diagnosis techniques were also used to detect myocardial metabolic changes in diabetic patients. McGavock et al. were able to detect the excessive storage of lipid in myocardium of patients with prediabetic stage using proton magnetic resonance spectroscopy, proposing this finding as an indicator of heart failure risk. However, they did not demonstrate a correlation between myocardial lipid content and cardiac function [16]. In addition, positron emission tomography was used to establish an association between myocardial metabolic derangement and early manifestation of diastolic function impairment with negative results [17].

Regarding serological biomarkers, natriuretic peptides and brain natriuretic peptide (BNP) in particular were proposed as suitable biomarkers for diastolic dysfunction in diabetic patients [18]. However, the utility of this molecule is controversial because plasma BNP rise is associated with excessive stretching of heart muscle cells, a condition associated with several cardiac diseases [19]. Troponins plasma concentration is associated with the magnitude of cardiomyocyte death, resulting in a biomarker of heart damage without any specificity of the cardiac disease etiology. In addition, troponins in plasma have a short half-life and are usually used to predict and establish heart failure [20].

At present, neither a laboratory test nor imaging techniques appear to be useful in diagnosing diabetic cardiomyopathy apart from diastolic dysfunction and to predict the risk of heart failure, excluding coronary artery disease, hypertension, or congenital heart failure [13]. Therefore, regarding the subclinical detection of diabetic cardiomyopathy, regulators of metabolic changes in the heart, as mentioned above, also present in biofluids, could be appropriate candidates as biomarkers. In the last 6 years, a large number of publications have been reported with promising results about the correlation of diseases manifestation and miRNAs (showing potential as a new class of biomarker) [21].

3. miRNAs Role in Diabetic Cardiomyopathy

As we previously described, changes in gene expression of key molecules involved in the pathogenesis of diabetic cardiomyopathy can be influenced by environmental factors, for instance, high fat diets, tobacco smoke, or epigenetic factors [22]. At present, epigenetic studies describe three mechanisms to link the type of exposure with cellular gene expression response: DNA methylation, histone modification, and miRNA expression [23].

MicroRNAs or miRNAs are small noncoding RNA molecules (≈22 nucleotides) which downregulate gene expression by a posttranscriptional mechanism controlling approximately 30% of all protein-coding genes of mammalian genome [24, 25]. During the past 7 years, researchers have identified several miRNAs and their specific mRNA targets altered in diabetic cardiomyopathy using experimental models at preclinic level, demonstrating the significant role of miRNAs in the progression of diabetic heart complication (Table 1). Human biopsies of diabetic heart showed an upregulation of miR-223 with an inhibition of *GLUT4* gene expression, reducing glucose uptake. This miRNA-mRNA

Table 1: Identification of miRNA involved in diabetic cardiomyopathy pathogenesis.

miRNA	Gene expression	Preselected miRNAs/screening method	Tissue source/experimental model	Target genes and/or pathophysiological effect	References
miR-1	↑	miRNA selected from [77, 78]	H9c2 cells exposure to high glucose levels	Block IGF-1 signal pathway inducing apoptosis	[33]
miR-1	↓	miR-1, miR-21, miR-133a, miR-499, miR-133b/1900 microRNAs approx. GeneChip miRNA arrays based on miRBase 17, Affymetrix	Mice heart/STZ-induced diabetic rat for 5 weeks (1 dose of 50 mg/Kg)	Junctin, which is involved in cardiomyocyte calcium handling	[34]
miR-320	↑	let-7e, miR-129, miR-291-5p, miR-320, miR-327, miR-333, miR-363-5p, miR-370, miR-494, miR-503, miR-664/274 miRNAs, microarray for miRNA based on miRBase 8, Exiqon	Myocardial microvascular endothelial cells/nonobese DMT2 animal model (Goto-Kakizaki rat)	IGF-1; angiogenic factor	[36]
miR-133a	↓	miR-1, miR-9, miR-16, miR-20, miR-23b, miR-24, miR-26a, miR-30a-5p, miR-30d, miR-93, miR-122a, miR-133a/b, miR-146a/b, miR-187, miR-197, miR-203, miR-207, miR-297, miR-299-5p, miR-320, miR-324-3p, miR-326, miR-335, miR-341, miR-345, miR-346, miR-62, miR-369-5p, miR-370, miR-371–miR-374, miR-422b, miR-431, miR-432, miR-467m, miR-483, miR-487a, miR-497, miR-500, and miR-518d/miRVana microarray for 486 miRNAs, Ambion microarray	Mice heart/STZ-induced diabetic mice for 2 months (1 dose of 150 mg/Kg)	Cardiac hypertrophy	[29]
miR-223	↓	TaqMan MicroRNA Assays Human Panel Early Access, for 155 different miRNAs, Applied Biosystems	Human heart/biopsies of NGT and DMT2 patients	GLUT4; glucose uptake	[26]
miR-373	↓	miR-1, miR-20a, miR-21, miR-24, miR-29, miR-142-3p, miR-143, miR-195, miR-199a-3p, miR-220b, miR-208a, miR-221, miR-373, miR-499-3p, miR-700, miR-705/CapitalBio Mammalian miRNA Array V4.0, based on miRBase 12, CapitalBio Corp.	Mice heart/STZ-induced diabetic mice for 2 months (1 dose of 150 mg/Kg)	Cardiac hypertrophy and myocardial fibrosis via mitogen-activated-protein kinase cascades pathway activation and RASA1, RAC1, TGFB3, and COL1A1 expression	[30, 31]

Table 1: Continued.

miRNA	Gene expression	Preselected miRNAs/screening method	Tissue source/experimental model	Target genes and/or pathophysiological effect	References
miR-141	↑	miR-141, miR-200c, miR-208b, miR-295/RT-PCR Array system for 376 miRNAs, SABiosciences	Mice heart/STZ-induced diabetic mice for 5 weeks (5 doses daily of 50 mg/Kg)	Slc25a3: regulator of the mitochondrial phosphate carrier expression, which is involved in ATP mitochondrial production	[38]
miR-30d	↑	miRNA selected for diverse papers reviewed in [35]	Rat heart/STZ-induced diabetic rat for 3 days (3 doses of 35 mg/Kg/day); neonatal rat cardiomyocyte exposure to high glucose levels	FOXO3a; induction of cardiomyocyte pyroptosis and cardiac inflammation	[35]
miR-34a	↑	miRNA selected from [79]	H9c2 cells exposure to high glucose levels	BCL-2; induction of apoptosis	[32]
miR-150	↓	miRNA selected from [80]	Neonatal rat cardiomyocyte exposure to high glucose levels	P300, which plays a role in cardiomyocyte hypertrophy	[28]
miR-301a	↑	miRNA selected from [81, 82]	Mice heart/db/db mice of 13-14 weeks of age	Kv4.2 channel; electrical remodelling	[37]
miR-451	↑	1300 miRNAs approx. Based on miRBase 19, miRNA microarray system 3D-Gene	Mice heart/obese mice fed with high fat diet for 20 weeks; neonatal rat ventricular cardiomyocytes exposure to palmitic acid	Cardiac hypertrophy through suppression of the LKB1/AMPK pathway	[39]

H9c2 cells: cardiac cell line derived from rat myocardium, STZ: streptozotocin, db/db mice: homozygous for diabetes spontaneous mutation in leptin receptor, NGT: normal glucose tolerance, and DMT2: diabetes mellitus type 2.

interaction was confirmed using neonatal rat cardiomyocyte [26]. El Azzouzi et al. reported that miR-199a/miR-214 cluster downregulated the peroxisome proliferator-activated receptor δ gene expression, which is a critical regulator of energy metabolism switch between fatty acid oxidation and glycolysis, impairing mitochondrial fatty acid oxidation [27]. Cardiomyocyte hypertrophy induced by exposing neonatal rat cardiomyocytes to high levels of glucose identified significantly reduced expression of three miRNAs (miR-150, miR-133a, and miR-373) involved in cardiac hypertrophy process [28–30]. Diao et al. identified sixteen microRNAs differentially expressed in hearts of DMT1 animal model induced by streptozotocin and proposed 4 gene targets (*Rasa1*, *Rac1*, *Tgfb3*, and *Col1A1*) associated with cardiac hypertrophy and myocardial fibrosis [31]. Upregulation of miR-34a and miR-1, induced by high glucose exposure, decreased *Bcl-2* and *Igf-1* gene expression, respectively, promoting apoptosis in H9c2 cells [32, 33]. Regarding cardiomyocyte Ca^{+2} handling during contractility-relaxation cycle, Yildirim et al. demonstrated that myocardial miR-1 downregulation produces an increase of junctin levels in streptozotocin-induced diabetic mice. Junctin is a component of the ryanodine receptor Ca^{+2} release channel complex in sarcoplasmic reticulum [34]. These data suggest that miR-1 has many target genes (e.g., *Igf-1* and *Junctin*) and its regulation could depend on the experimental model, indicating the nonspecificity of this miRNA in diabetic cardiomyopathy. Upregulation of miR-30d promoted cardiomyocyte pyroptosis (the proinflammatory programmed cell death) in a DMT1 animal model, via the repression of foxo3a and apoptosis repressor with caspase recruitment domain expression and, consequently, the activation of caspase-1 and secretion of proinflammatory cytokines (IL-1β and IL-18) [35]. Using myocardial microvascular endothelial cells from a nonobese DMT2 animal model (Goto-Kakizaki rat), upregulation of miR-320 was reported that reduced *Igf-1* gene expression decreasing angiogenic response to diabetes-derived microvascular injury [36]. miR-301 upregulation alters the voltage-gated potassium channel in diabetic heart of *db/db* mice (animal model for DMT2), generating an electrical remodelling [37]. miR-141 upregulation in heart of diabetic mice (induced with streptozotocin) reduced the gene expression of *Slc25a3* (inner mitochondrial phosphate transporter), resulting in a decreased ATP production [38]. Recently, Kuwabara et al. showed that miR-451 exacerbates lipotoxicity in cardiac myocytes and cardiac hypertrophy in obese mice fed with high fat diet for 20 weeks (a physiological obese animal model for a prediabetic state of DMT2) through the direct interaction with Cab39, scaffold protein of liver kinase B1 (LKB1), which suppress the LKB1/AMPK pathway. As AMP-activated protein kinase (AMPK) is a major cellular response of energy availability, its suppression reduced the cardiac functional reserve [39]. We summarised the miRNAs in pathological mechanism of diabetes cardiomyopathy in Table 1.

4. miRNAs as Biomarkers

Besides the intracellular functions of miRNAs, recent studies demonstrated that miRNAs are also paracrine mediators of cell-to-cell communication transported via microvesicles called exosomes. Cardiac cell communication via exosomes in healthy and pathological conditions is an emerging research field for understanding the development of cardiac diseases [40]. Malik et al. published that oxidative stress or hypoxia/reoxygenation transition stimulated cardiomyocyte to secrete exosomes containing mRNAs and miRNAs [41]. In addition, external cell signalling, as growth factor stimulation, can regulate the transcriptional contents of secreted exosomes in cardiomyocytes [42]. Waldenström et al. reported that exosomes released from cardiomyocytes affect gene expression in fibroblast [43]. Reciprocally, cardiac fibroblast was found to release miR-21 via exosomes and was associated with cardiomyocyte hypertrophy [44]. According to the current scientific evidences, both miRNA composition and quantity could be considered a reflex of metabolic or differentiated state of exosome-producing cells. Circulating exosomes can be identified in all biofluids, particularly in plasma or serum [45], and, therefore, becoming an attractive tool for analytical studies and subsequent diagnosis of the diseases.

Moreover, circulating miRNAs in plasma are also transported and delivered to recipient cells on circulating high-density lipoprotein (HDL) [46]. HDL has a crucial role in the progression of cardiolipid-metabolism modifications occurring in diabetic cardiomyopathy development; for instance, in cardiomyocyte FFA oxidation via AMP-kinase activation and its accumulation prevents lipotoxicity in diabetic heart [47]. The discovery of miRNA participation in the regulation of lipoprotein synthesis, composition, transport, and degradation has provided new targets for therapy to improve the cardioprotective properties of HDL, particularly in coronary artery disease due to HDL regulation of cholesterol homeostasis [48]. Taking that into account, circulating miRNA in HDL complex could also be an indicator of metabolic changes in lipid-tissue accumulation diseases such as diabetic cardiomyopathy.

A third class of circulating miRNAs bind to soluble proteins called Argonautes, which are key players in all small-RNA-guided gene silencing processes [49]. Biophysics studies demonstrated that extracellular miRNA circulating in the bloodstream is remarkably stable, in spite of being presented in an RNAse-rich environment, due to its encapsulation in microvesicles/exosomes or its binding to the proteins [50]. This known stability of miRNA-exosomes and miRNA-protein complex is another relevant characteristic suitable for biomarkers.

5. Evidences of miRNA Profiling for Diabetic Cardiomyopathy

Identification of miRNA associated with diabetes and its complications in humans escalated with the possibility of screening multiple miRNAs simultaneously using profiling techniques including microarray and Next Generation Sequencing (NGS), in damaged or injury tissues of diabetes animal models due to miRNA-mRNA interaction found to be conserved between most mammalians [51]. The gene target identification of the selected miRNAs has been

studied *in vitro* using neonatal rat cardiomyocytes where the miRNA-mRNA interaction is correlated with changes in cell phenotype. The process of gene targets identification is accelerated by bioinformatics techniques such as TargetScan, DIANA-mirExTra, PITA, miRNADA, miRDB, and PICTAR that reduce the number of possibilities to test experimentally [52–54]. In this context a good resource that integrates all these tools is miRWalk database that allows choosing between predicted and validated experimental miRNA-mRNA interactions [55].

The discovery of placental miRNA in maternal plasma in 2008 [56], along with the nascent hypothesis of its role in the cell-to-cell communication, has promoted the study of miRNAs as biomarkers in cancer through the specific secretion via exosomes by tumour cells [21]. In the same year, Lawrie et al. reported elevated levels of tumour-associated miRNAs in serum of patients suffering from diffuse large B-cell lymphoma [57]. Several clinical studies are also performed proposing use of miRNA in early diagnosis of chronic illness including cardiovascular and neurodegenerative diseases [58–60]. Therefore, pharmaceutical companies are very interested in developing a diagnostic kit for several types of cancer and chronic diseases [61].

According to the last version (21st) of miRNA database (miRBase) [62], there are 1881 human sequences identified and the list is still growing; Friedländer et al., employing an innovative computational method, reported 2469 novel human miRNA candidates [63]. Therefore, researchers have used two experimental strategies to find the "needle in a haystack": using omics approaches (microarray or NGS) and/or preselecting miRNAs based on previous finding reported in animal models (Table 2). In this context, NGS offers several advantages; for instance, it does not require the knowledge of either miRNA target or specific probes or primers to discover new miRNAs [64]. Regarding diabetes, since 2010 several differentially regulated miRNAs have been identified in human biofluids from patients of impaired glucose tolerance (IGT)/impaired fasting glucose (IFG), DMT2 in comparison to healthy controls (Table 2).

Reviewing the articles published in the last five years, we have concluded that there is not an extensive overlap in the results of miRNAs identification associated with different conditions of diabetes patients. Only miR-126 and miR-144 have been proposed as biomarkers for diagnosing diabetes in more than one study [65–68]. Although these clinical studies used the same range of values for glucose tolerance test (the most important parameter for patients classification), there are other factors that could explain the differences: (1) clinical characteristics including obesity, age, year after diagnosis, and lipid profile [69]; (2) treatment with hypoglycaemic drugs such as metformin [66]; (3) ethnical origin such as Iraqis versus Swedes [70]; (4) technical aspects: types of biofluids, differences in miRNA microarrays companies (quantity and type of miRNA tested), and miRNA normalization methods.

Regarding diabetic cardiomyopathy, diagnosed patients with diastolic dysfunction share many clinical characteristics with diabetic patients reported in the clinical study for the identification of miRNAs related to diabetes (Table 2). However, at present there are no clinical trials reporting circulating miRNAs as a candidate for diabetic cardiomyopathy diagnosis. On the other hand, there is no concordance between the miRNA identified with diabetic cardiomyopathy in animal models and those identified in human biofluids, except miR-34a and miR-30d, which were identified first in human plasma and then their mechanism of action was studied at a preclinical level [32, 35]. The diabetic complications have a slow but progressive negative manifestation in the target organs (kidney, liver, heart, and retina) with respect to the apparent stability of metabolic parameters (fasting glucose and glucose tolerance test). As diabetes is a multifactorial disease, care should be taken when enrolling the patients, by following strict definitions of the clinical characteristic of the diabetic complications. In a cross-sectional study where DMT1 patients were classified in three groups according to their level of renal dysfunction by eGFR, good renal function $\geq$ 30 mL/min of creatinine clearance, renal failure < 30 mL/min of creatinine clearance, and healthy control, four miRNAs (miR-181, miR-326, miR-126, and miR-573-3p) were identified in plasma that could be useful to predict the development of diabetic nephropathy [71].

The use of disease animal models is a powerful tool to select circulating miRNAs candidate for biomarker as it allows establishing a correlation between miRNAs associated with specific injured organ and the biofluid from early to advanced stages of disease development. For instance, Bellinger et al. found a concordance between the expression of miR-714, miR-1188, miR-1897-3p, miR-877, and miR-1224 and progression of acute kidney injury reflected in the plasma of a mouse model, proposing them as a promising predictor of kidney injury [72]. Acharya et al. reported serum miRNA signatures that predict the impact of radiation in animals that were exposed to sublethal and lethal doses of radiation, 24 hours after exposure [73], and Rotkrua et al. selected circulating miRNAs (miR-103, miR-107, miR-194, and miR-210) as biomarkers for early detection of diffuse-type gastric cancer using a mouse model and compared expression of these miRNAs in tumour tissue and serum samples [74]. The strategy of using diabetic cardiomyopathy animal models to find a corelation of miRNAs expression between myocardium and biofluids would not only provide relevant information but also accelerate miRNA identification [8, 75].

Regarding methodological aspects, it has been established that using different evaluation techniques may yield variations in the end results. For this reason, in the last years many methodological studies have been published comparing different laboratory procedures: (1) sample collection, (2) total miRNA isolation, (3) miRNA profiling methods, including qRT-PCR, miRNA microarrays (GeneChip and miRCURY LNA), and NGS, and (4) criteria of data analysis including miRNA normalization method (spike-in or internal miRNA). Further, alternatives in miRNA procedures were elaborately discussed in an excellent review of Moldovan et al. [76].

In a future perspective, a consistent miRNA profiling is not enough to diagnose a disease; informatics algorithms such as naïve Bayes classifier, J48 Decision Trees, and support vector machines are also necessary to identify the best miRNA profiling (considering all clinical characteristics) to discriminate between diabetic patients, which of them

Table 2: Identification of miRNAs identified in human biofluids as potential biomarkers for diabetes mellitus type 2.

Proposed miRNAs as biomarkers	Gene expression (diabetic versus healthy patients)	Type of samples/normalized method	Preselected miRNAs from screening method or from preclinical studies	Type of clinical study/experimental groups (number)	Geographic location	References
miR-126	↓	Plasma/RT-qPCR, miR-454	miR-15a, miR-20b, miR-21, miR-24, miR-29b, miR-126, miR-150, miR-191, miR-197, miR-223, miR-320, miR-486/Human TaqMan MicroRNA Arrays for 377 miRNAs, Applied Biosystems	Prospective population-based study/NGT (580) IFG-IGT (162) DMT2 (80)	Bruneck, Italy	[65]
miR-144	↑	Blood/RT-qPCR	miR-144, miR-146a, miR-150, and miR-182 selected from blood liver, pancreas, skeletal muscle, and adipose tissue of rat fed with high fat diet (2 weeks) and STZ administration (40 mg/Kg, ip)/microarray for miRNA based on miRBase 11, Exiqon	Cross-sectional study/NGT (15) IGT-IFG (14) DMT2 (21)	Singapore, Singapore	[68]
miR-9 miR-29a miR-30d miR-34a miR-124a miR-146a miR-375	↑	Serum/RT-qPCR RNU6B	miRNAs selected from [83]	Cross-sectional study/NGT (19) IGT-IFG (19) DMT2 (18)	Jinan, China	[79]
miR-126 miR-140-5p miR-142-3p miR-195 miR-423-5p	↓	Plasma/RT-qPCR miR-106a miR-146a miR-19b miR-223	miR-125b, miR-126, miR-130b, miR-140-5p, miR-142-3p, miR-192, miR-195, miR-222, miR-423-5p, and miR-532-5p/TaqMan Array Human MicroRNAs v2.0, for 377 miRNAs, Life Technologies	Pilot study/NGT (6) DMT2 (6) Cross-sectional study/NGT (45) DMT2 (48)	Girona, Spain	[66]
miR-138, miR-376a miR-503	↓	Serum/RT-qPCR miR-191 miR-423-3p	miR-15b, miR-25, miR-27b, miR-101, miR-138, miR-150, miR-205, miR-376a, miR-432-5p, miR-500a, miR-503, and miR-942/Human Panels I and II containing 742 miRNAs, Exiqon	Cross-sectional study/NGT (20) Obese (20) DMT2 (13) Obese + DMT2 (16)	Madrid, Spain	[69]

Table 2: Continued.

Proposed miRNAs as biomarkers	Gene expression (diabetic versus healthy patients)	Type of samples/normalized method	Preselected miRNAs from screening method or from preclinical studies	Type of clinical study/experimental groups (number)	Geographic location	References
miR-21 miR-210 (plasma) miR-126 (urine)	↑ ↓	Plasma, urine/RT-qPCR	miRNA-21, miR-126, and miR-210/miRNAs selected from [65, 84, 85]	Cross-sectional cohort study with paediatric patients NGT (79) DMT1 (68)	London, United Kingdom	[86]
miR-126	↓	Serum/RT-qPCR Cel-miR-39	miRNA selected from [65]	Cross-sectional cohort study/NGT (138), IGT/IFG (157), DMT2 (160)	Harbin, China	[67]
miR-24 miR-29b miR-144 (for Swedes)	↑	Plasma/RT-qPCR miR-425	miR-15b, miR-20, miR-21, miR-24, miR-28-3p, miR-29b, miR-126, miR-144, miR-150, miR-191, miR-197, miR-223, and miR-302a, miR-486-5p selected according to miRNA described in [65, 68]	Cross-sectional study/NGT-Iraq (65) NGT-Sweden (54) DMT2-Iraq (19) DMT2-Sweden (14)	(Sweden and Iraqi population) Malmö, Sweden	[70]
miR-101 miR-375 miR-802	↑	Serum/RT-qPCR Cel-miR-39	miR-101, miR-335, miR-375, and miR-802 selected by preselected miRNAs from heart, pancreas, white adipose tissue, and other tissues of obese mice fed with high fat diet of 20 weeks of age were obtained by sequence analysis Illumina	NGT (49) DMT2 (155)	Okayama, Japan	[87]

NGT: normal glucose tolerance, IGT-IFG: impaired glucose tolerance-impaired fasting glucose, DMT2: diabetes mellitus type 2, STZ: streptozotocin, and ip: intraperitoneal.

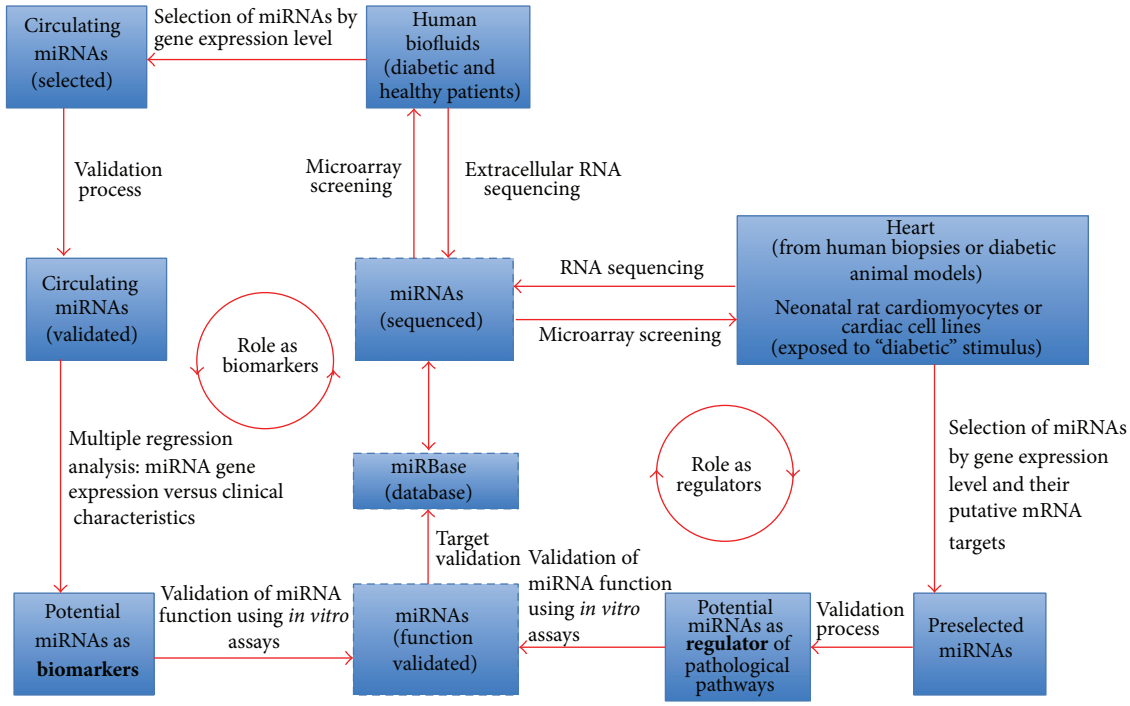

Figure 1: Experimental strategies for circulating microRNA identification as potential biomarkers.

have a high risk of developing diabetic cardiomyopathy. A summary of the experimental strategies for the identification of circulating miRNAs as biomarkers is described in Figure 1.

6. Conclusions

Diabetic cardiomyopathy progresses slowly and silently and is diagnosed (with the current detection procedures) only when the heart manifests a certain grade of dysfunction. This subclinical state of the illness becomes even more critical after an ischemic episode as it reduces the possibility to rescue the heart function to normal levels. At present, scientific evidence of the potential use of circulating miRNAs as biomarkers for cardiovascular diseases is increasing every day and is extensively backed up by the studies carried out by research groups of public and private institutions. The identification of miRNAs in diabetic patients "without" any secondary complications is relevant as it would allow narrowing down the miRNAs associated with specific diabetic complication. Regarding diabetic cardiomyopathy, the early detection using miRNAs biomarker could serve to intensify the antidiabetes treatment and cardioprophylactic therapies in patients with high risk of diabetes-derived diastolic dysfunction before it appears.

Conflict of Interests

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

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