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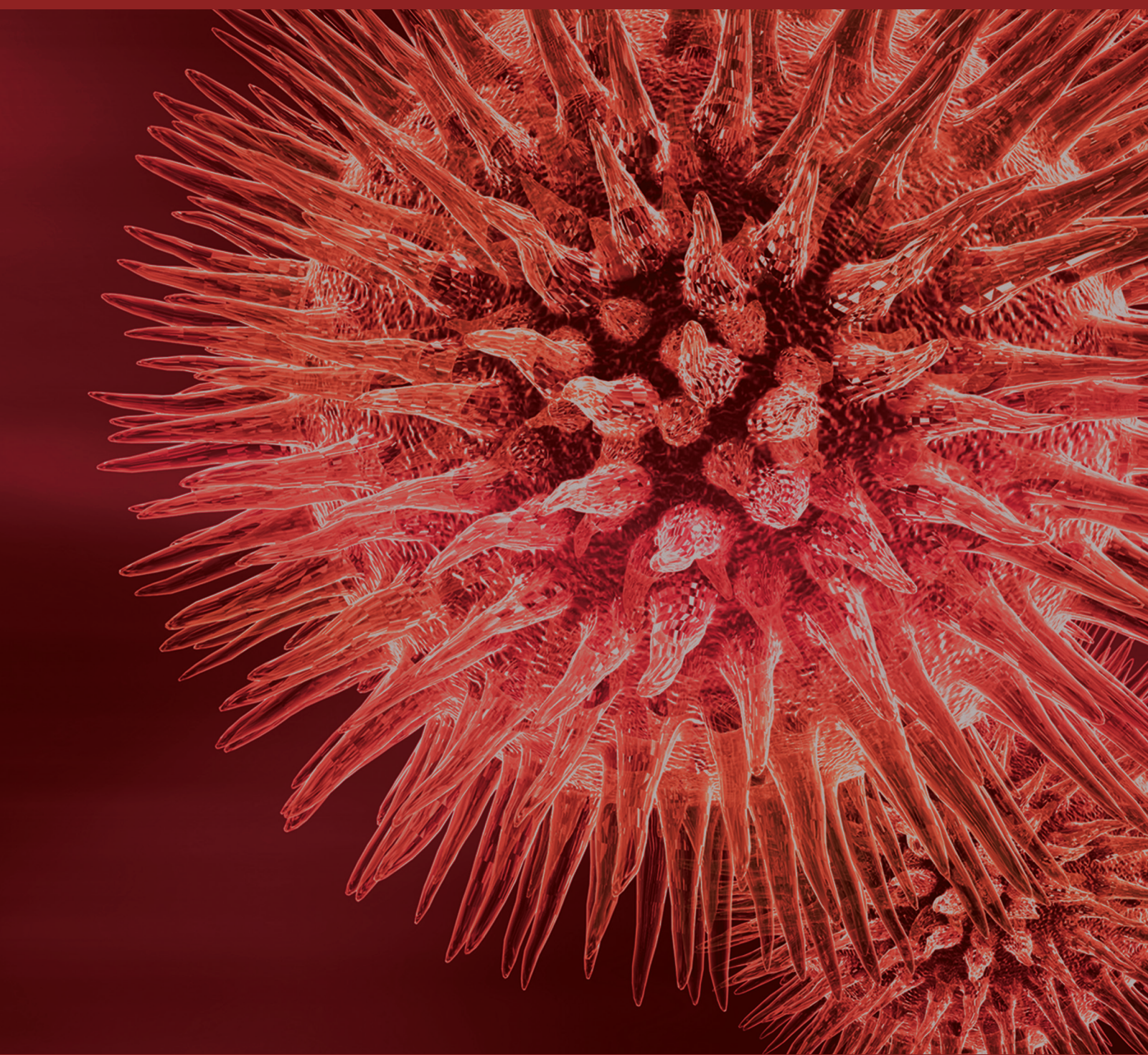
Cardiac Complications of Diabetes

Lead Guest Editor: Charbel Abi Khalil
Guest Editors: Jassim Al Suwaidi, Marwan Refaat, and Kamel Mohammedi



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Editorial

Cardiac Complications of Diabetes

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Type 2 diabetes (T2D) is reaching epidemic proportions through most regions of the world. In a large population health study, the global age-standardized diabetes prevalence increased from 4.3% in 1980 to 9.0% in 2014 in men, and from 5.0% to 7.9% in women [1]. Potential causes for the higher incidence of T2D worldwide are increased survival of the elderly as well as epidemic bursts of obesity and sedentary lifestyle at all age ranges [2]. Diabetes is associated with a higher incidence of cardiovascular events, which results in a higher rate of mortality [3]. In patients without a history of cardiovascular disease, the 7-year risk of myocardial infarction (MI) is as high as 20% in patients with T2D as compared to only 3.5% in people without diabetes [4]. T2D also increases the risk of ischemic stroke by 35% as demonstrated in the large international case-control INTERSTROKE study [5] and the risk of peripheral artery disease (PAD) by 2- to 4-fold [6].

However, diabetes-related cardiac complications have a distinct pathophysiological background and clinical presentation. In order to understand the deleterious effect of hyperglycemia on the cardiovascular system, we propose in this special issue to highlight on all aspects of diabetes and cardiovascular diseases' interaction: epidemiology, pathophysiology, clinical manifestations, diagnosis, and treatment in 4 reviews and 6 original articles.

In their review, D. Huang et al. revisited the basic pathophysiological and epidemiological data regarding macrovascular complications in patients with diabetes and prediabetes. In fact, hyperglycemia induces low-grade inflammation and triggers the release of reactive oxygen species, which activates several pathways involved in endothelial dysfunction. Among those pathways, the formation of advanced glycation end-products (AGEs) plays a major role in initiating cardiovascular complications of diabetes. In this issue, A. Guerin-Dubourg reported that plasma concentrations of AGEs, including fructosamine, glycated albumin, and fluorescent ischemia-modified albumin, were increased in diabetic patients with established cardiovascular complications. N. El-Najjar et al. showed that hyperglycemia also induces calcium (Ca^{2+}) signaling disruption in vascular smooth cells, mainly by inhibiting the passive endoplasmic reticulum Ca^{2+} leak and the sarcoplasmic reticulum Ca^{2+} -ATPase.

Diagnosing cardiac complications of diabetes can be challenging since clinical manifestations can be sometimes silent, especially at early stages. However, considering all patients with diabetes as a coronary artery disease-risk equivalent represents a strain on the healthcare system and exposes patients to unnecessary invasive exams. A. I. Guaricci et al. concluded in their systematic review of asymptomatic coronary atherosclerosis assessment that cardiac computed tomography angiography might be an adequate preliminary noninvasive test that helps explore the atherosclerotic diffusion and establish the risk of future coronary events in patients with type 2 diabetes.

Mortality in patients with diabetes is a serious concern. However, little is known in elderly polyvascular patients with T2D. J. L. Clua-Espuny reports in this special edition that the prevalence of T2D in those complex patients is above 50%. Interestingly, diabetes per se does not increase the mortality risk in this particular population, but the presence of other comorbidities such as cognitive impairment, lack of aspirin treatment, and the presence of heart failure does. S. G. Al-Kindi et al. tested the prognostic utility of a novel, easy-to-use, biomarker: the red cell distribution width (RDW). In fact, a high RDW is associated with a higher cardiovascular mortality risk, independent to other diabetes comorbidities.

Individuals with T2D are predisposed to arrhythmias, including atrial fibrillation. In their retrospective analysis of direct current cardioversion (DCCV) data, H. Soran et al. showed that diabetes was an independent risk factor for failure of DCCV in atrial fibrillation patients. Further analysis revealed that HbA_{1C} was also a predictor for an immediate failure, on top of conventional factors such as the atrial size, the left ventricular ejection fraction, and previous DCCV failures.

Finally, 2 review papers tackled the treatment of T2D and cardiovascular outcome. M. P. Nasrallah et al. nicely summarized all trials conducted to establish either the safety or protection of antidiabetic drugs, from the old UKPDS and STOP-NIDDM to the recent SGLT2 inhibitors RCTs. D. von Lewinski et al.'s review focused on the relationship between new antihyperglycemic drugs and heart failure (HF), in the light of new studies such as EMPA-REG trial showing a decrease in HF mortality in patients using empagliflozin, which is opposed to other studies such as SAVOR-TIMI that suggested an increase in HF risk with glitazones.

It is expected that T2D will impose an enormous strain on healthcare in the future and result in mortality excess of millions around the world. The latter can only be partially reversed if education, prevention, early diagnosis, and appropriate treatment of cardiovascular complications are strictly applied.

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Charbel Abi Khalil
Jassim Al Suwaidi
Marwan Refaat
Kamel Mohammedi

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Research Article

Risk Factors for Failure of Direct Current Cardioversion in Patients with Type 2 Diabetes Mellitus and Atrial Fibrillation

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Introduction. Type 2 diabetes mellitus (T2DM) is a well-recognised risk factor for cardiovascular disease and the prevalence of atrial fibrillation (AF) is higher among patients with T2DM. Direct current cardioversion (DCCV) is an important management option in persistent AF. We sought to determine independent risk factors for immediate and short-term outcomes of DCCV for treatment of AF in patients with T2DM. *Methods*. Retrospective outcome analysis of DCCV for persistent AF in 102 T2DM patients compared with 102 controls. *Results*. DCCV was successful in 68 (66.6%) people with T2DM compared to 86 (84.3%) in the control group ($P = 0.003$). After initial successful cardioversion, only 38 (37.2%) T2DM patients remained in sinus rhythm compared to 63 (61.8%) in the control group ($P = 0.007$) at a median follow-up of 74.5 days (IQR 69.4–77.4). Multiple logistic regression analysis showed that the presence of T2DM ($P = 0.014$), digoxin use ($P = 0.01$), statin use ($P = 0.005$), left-atrial size ($P = 0.01$), and LV ejection fraction ($P = 0.008$) were independent risk factors for immediate DCCV failure. T2DM ($P = 0.034$) was an independent risk factor for AF relapse. Among patients with T2DM, previous DCCV ($P = 0.033$), digoxin use ($P = 0.035$), left-atrial size ($P = 0.01$), LV ejection fraction ($P = 0.036$), and HbA1c ($P = 0.011$) predicted immediate failure of DCCV whilst digoxin use ($P = 0.026$) was an independent risk factor for relapse of AF. *Conclusion*. T2DM, higher HbA1c, digoxin treatment, and structural and functional cardiac abnormalities are independent risk factors for immediate DCCV failure and AF relapse.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a well-recognised risk factor for cardiovascular disease (CVD) [1]. The major manifestations of CVD are mainly macrovascular, but changes in the coronary microcirculation are well documented among patients with T2DM [2]. The prevalence of atrial fibrillation (AF), a common feature of CVD, is higher among patients with diabetes, when compared to patients without diabetes [3]. AF also contributes to increased morbidity and mortality in patients with T2DM [3, 4]. Though the debate between rate and rhythm control rages on [5], direct current cardioversion (DCCV) is still rooted within clinical guidelines as an important management option for AF especially in those with symptomatic and persistent AF [6]. There is an increasing recognition that patients who have diabetes and

atrial fibrillation have a greater risk of ischaemic stroke and complications associated with anticoagulation. The success rates of both ablation and cardioversion are lower in those with diabetes [7]. In addition, modulation of the renin angiotensin system [8] and therapy with statins [9] may determine the risk of AF recurrence. We have previously reported that patients with T2DM are less responsive to DCCV; however, that cohort lacked robust control data [10]. We have therefore undertaken a case-control study to determine the independent risk factors for the immediate success rate and relapse following DCCV in patients with AF.

2. Patients and Methods

This was a retrospective case-control study in patients with persistent AF (pAF) attending the Cardiology Department for DCCV in a large district general hospital in Wirral, United Kingdom. We identified patients who had undergone DCCV between October 2001 and April 2007 ($n = 624$) using the hospital's electronic database. Relevant risk factor data were extracted from the medical records. The study was approved by the Wirral Research Ethical Committee.

Inclusion Criteria. All adult patients with documented pAF who underwent DCCV from October 2001 to April 2007 were included.

Exclusion Criteria. Patients with cardiac valvular disease (other than mild mitral and tricuspid valve regurgitation), rheumatic heart disease, previous heart valve surgery, congenital heart disease, patients with AF as a consequence of acute myocardial infarction (MI) or cardiac surgery, significant chronic kidney disease (defined as eGFR less than 60 ml/min/1.73 m^2), AF secondary to thyrotoxicosis, patients who reverted to sinus rhythm (SR) before DCCV, and patients in whom relevant data were missing from the notes were excluded from the study. A total of 45 patients were excluded: 14 had valvular heart disease, two had history of heart valve replacement, three had AF after coronary artery bypass grafting, eight reverted to SR before attempted DCCV, two had AF secondary to thyrotoxicosis, five developed AF in the course of acute MI, and 13 were excluded because of missing data (Figure 1).

Of the 364 eligible patients, 102 had T2DM and of the 262 patients without T2DM 102 age and gender matched patients were selected as the control group for this study (Table 1).

All patients were anticoagulated for at least 6 weeks prior to cardioversion. International normalized ratio (INR) was checked at least weekly for the preceding 4 weeks prior to cardioversion and the dose of warfarin was adjusted to maintain the INR between 2.0 and 3.0 (target INR 2.5). Digoxin was stopped in all patients 48 hours before cardioversion and not recommenced in patients who had successful DCCV. Thyroid function tests, urea and electrolytes, full blood count, and cholesterol were checked in all patients. If amiodarone, sotalol, or flecainide were prescribed, patients continued on these until reviewed in clinic. All patients were reviewed 3 to 4 hours after DCCV (prior to discharge) and subsequently

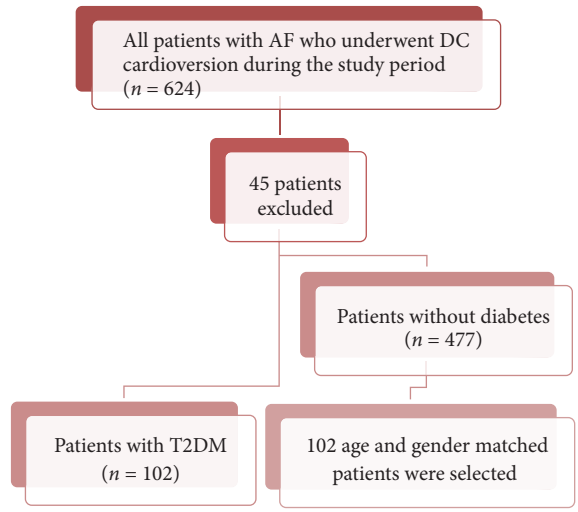

Figure 1: Study design and patient selection.

in the outpatient clinic. Clinical examination and a 12-lead electrocardiogram (ECG) were performed at each review.

Successful DCCV was defined by an ECG 3 hours after DCCV showing sinus rhythm. Persistent AF was defined as episodes that failed to self-terminate spontaneously and lasted for longer than 7 days but could be converted to SR with pharmacological or electrical cardioversion. The duration of AF was the period (in weeks) from the first day of diagnosis of the index atrial fibrillation supported by ECG to the day of DCCV. In patients who had a successful cardioversion, the arrhythmia status on the day of first outpatient follow-up was documented to assess differences between the two groups. All patients with a documented history of hypertension or three recent blood pressure readings greater than 160/90 mmHg were regarded as having hypertension. All patients on lipid-lowering treatment or with a total serum cholesterol measurement greater than 5.2 mmol/l were regarded as having hyperlipidaemia. In patients with diabetes, a mean HbA1c of the three most recent measurements was used. HbA1c values were measured by affinity chromatography using a commercial kit (BioRad, UK; nondiabetic range 22–40 mmol/mol [4.2–5.8%]). Echocardiograms were performed in the Department of Cardiology, Wirral University Hospitals NHS Foundation Trust (using Philips-Sonos 5500 and Philips-Sonos 5400). All echocardiogram operators had British Society of Echocardiography accreditation. An estimate of left ventricular ejection fraction (LVEF) was made using automated software. Left ventricular hypertrophy (LVH) was diagnosed using echocardiography by measuring the thickness of the intraventricular septum and posterior wall. Using an anteroapical position, monophasic DCCV was performed on an elective basis with a Hewlett Packard device. One hundred joules was used as the initial energy current and titrated according to response to a maximum of 360 J.

Statistical analysis SPSS v22.0 was used to analyse the data. Data were expressed as frequency and percentage for categorical data and mean ± SD for continuous data. We used recurrence of AF at the first outpatient follow-up as the dependent outcome. Univariate model with a cut-off of

Table 1: Baseline characteristics of patients. Data are mean (SD). AF, atrial fibrillation; DC, direct current; CAD, coronary artery disease; PAD, peripheral arterial disease; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; LA, left atrium; LV, left ventricle.

Characteristics	Patients without T2DM ($n = 102$)	Patients with T2DM ($n = 102$)	P
Age, years	69.4 ± 7.8	68.9 ± 7.6	NS
Gender ratio, M : F	0.73	0.73	NS
Duration of AF prior to DC cardioversion, weeks	14.7 ± 8.8	20.3 ± 18.8	0.007
Previous DC cardioversion, n (%)	6 (5.9)	3 (2.9)	NS
History of smoking, n (%)	2 (2.0)	8 (7.8)	0.052
Preexisting atherosclerotic disease (CAD, stroke, and PAD), n (%)	45 (44.1)	50 (49.0)	NS
Obstructive airways diseases, n (%)	11 (10.8)	8 (7.8)	NS
Hyperlipidaemia, n (%)	36 (35.3)	42 (41.2)	NS
History of alcohol excess, n (%)	4 (3.9)	2 (2.0)	NS
ACE-inhibitor or ARB use, n (%)	40 (39.2)	63 (61.8)	0.001
Amiodarone use, n (%)	46 (45.1)	40 (39.2)	NS
Flecainide use, n (%)	1 (1.0)	2 (2.0)	NS
Sotalol use, n (%)	4 (3.9)	4 (3.9)	NS
Beta-blocker use, n (%)	33 (32.4)	27 (26.5)	NS
Calcium channel blocker use, n (%)	23 (22.5)	33 (32.4)	NS
Digoxin use, n (%)	29 (28.4)	36 (35.3)	NS
Statin use, n (%)	29 (28.4)	32 (31.4)	NS
LA size, cm	4.3 ± 0.8	4.3 ± 0.7	NS
LV ejection fraction, %	56.2 ± 11.8	54.0 ± 12.4	NS
Presence of LV hypertrophy, %	14 (13.7)	10 (9.8)	NS
1st follow-up visit after DC cardioversion, days	75.2 ± 6.9	74.9 ± 7.5	NS

$P < 0.05$ was used to identify potentially significant factors. Multiple regression analyses using the factors identified above were used to identify independent risk factors for recurrence of AF following successful DC cardioversion. A P value < 0.05 was statistically significant.

3. Results

102 patients with T2DM with a mean diabetes duration of 5.5 (95% CI: 4.6–6.4) years and 102 age and gender matched control patients without T2DM underwent DCCV for nonvalvular AF (Table 1). Apart from the duration of AF prior to DCCV and use of renin angiotensin system (RAS) inhibitors, there were no significant differences between the two groups.

DCCV resulted in immediate (prehospital discharge) success in 75.5% of all patients. Of the 102 patients with T2DM, 68 (66.6%) achieved immediate cardioversion to sinus rhythm compared to 86 (84.3%) of 102 patients without T2DM (OR: 0.372, 95% CI: 0.19–0.73, and $P = 0.003$). The median time to the 1st follow-up visit was 75 [95% CI: 74.0–76.0] days; of the 68 patients with T2DM who had achieved immediately successful DCCV, 38 (55.9%) had maintained sinus rhythm compared to 63 of 86 (73.3%) patients without T2DM [OR: 0.398, 95% CI: 0.203–0.730, and $P = 0.007$] (Figure 2).

3.1. Multiple Regression Analysis. After adjusting for age, gender, duration of AF, previous DCCV, smoking history, preexisting atherosclerotic disease, dyslipidaemia, alcohol excess, RAS inhibitor usage, any heart-rate limiting agent usage, and presence of LVH, T2DM (β: −0.144, 95% CI of β: −0.259 to −0.029, $P = 0.014$), use of digoxin (β: −0.162, 95% CI of β: −0.285 to −0.040, and $P = 0.01$), statin use (β: −0.204, 95% CI of β: −0.347 to −0.062, and $P = 0.005$), left-atrial size (β: −0.103, 95% CI of β: −0.181 to −0.025, and $P = 0.01$), and LVEF (β: 0.006, 95% CI of β: 0.002 to 0.011, and $P = 0.008$) independently influenced attainment of SR immediately after DCCV (Table 2). A further model which also included the time between DCCV and the first follow-up visit as well as the aforementioned factors found T2DM (β: −0.186, 95% CI of β: −0.357 to −0.014, and $P = 0.034$) to independently and inversely influence the persistence of SR at the 1st visit following DCCV.

Table 2: Regression model assessing factors affecting attainment of sinus rhythm immediately after DCCV. DCCV, direct current cardioversion; LA, left atrium; LVEF, left ventricular ejection fraction; T2DM, type 2 diabetes mellitus.

Variables	β-Coefficient	P
T2DM	−0.144	0.014
Digoxin use	−0.162	0.010
Statin use	−0.204	0.005
LA size	−0.103	0.010
LVEF	0.006	0.008

In patients with T2DM, after adjusting for age, gender, duration of AF, smoking history, preexisting atherosclerotic disease, obstructive airways disease, dyslipidaemia, alcohol excess, use of statins, RAS inhibitors, and rate limiting agents,

Table 3: Regression model assessing factors affecting attainment of sinus rhythm immediately after DCCV in patients with T2DM. DCCV, direct current cardioversion; HbA1c, glycated haemoglobin; LA, left atrium; LVEF, left ventricular ejection fraction; T2DM, type 2 diabetes mellitus.

Variables	β-Coefficient	P
Previous DCCV	−0.558	0.033
Digoxin use	−0.206	0.035
LA size	−0.184	0.010
LVEF	0.007	0.036
HbA1c	−0.104	0.011

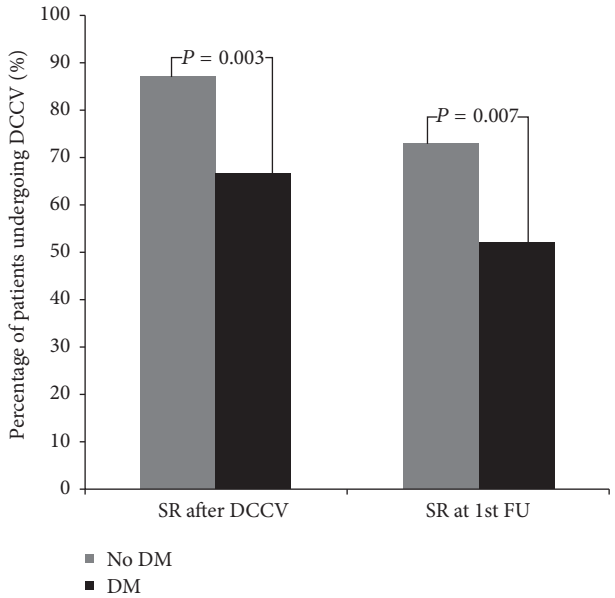

Figure 2: The percentage of patients with and without T2DM in sinus rhythm immediately after DC cardioversion and at the first follow-up visit. There were a higher percentage of patients achieving sinus rhythm immediately after DC cardioversion without diabetes than with T2DM, ($P = 0.003$) and at the first follow-up visit ($P = 0.007$).

presence of left ventricular hypertrophy and microvascular disease (as defined by presence of any degree of retinopathy or neuropathy documented in the medical notes as part of annual screening and assessment, nephropathy defined as documented microalbuminuria with urinary albumin: creatinine ratio of >3 mg/mmol on two occasions), previous DCCV (β: −0.558, 95% CI of β: −1.071 to −0.46, and $P = 0.033$), use of digoxin (β: −0.206, 95% CI of β: −0.397 to −0.015, and $P = 0.035$), left-atrial size (β: −0.184, 95% CI of β: −0.324 to −0.045, and $P = 0.01$), LV ejection fraction (β: 0.007, 95% CI of β: 0.001 to 0.014, and $P = 0.036$), and HbA1c (β: −0.104, 95% CI of β: −0.183 to −0.025, and $P = 0.011$) independently influenced attainment of sinus rhythm immediately following DCCV (Table 3). A further model which included the time between DCCV and the first follow-up visit along with the aforementioned factors found digoxin use (β: −0.413, 95% CI of β: −0.774 to −0.051, and $P = 0.026$) to independently and inversely influence persistence of sinus rhythm at the 1st visit following DCCV.

4. Discussion

Conceptually, consequences of AF can be mostly ameliorated by attainment of sinus rhythm, though this has been debated [11]. When chemical cardioversion fails electrical cardioversion may be attempted depending on physician and patient preference [6]. In the present study the overall immediate prehospital discharge success rate of DCCV in our patients was 75.5%, which is comparable to previous reports [12]. Possible reasons for failure to attain and maintain sinus rhythm have been discussed by Frick et al. [12], but their list does not include diabetes. Indeed, there are very limited data regarding success rate of DC cardioversion in patients with diabetes. Thus in a small cohort of 48 patients, where the efficacy of atorvastatin in preventing the recurrence of AF was investigated, diabetes was found to be a significant risk factor influencing the risk of recurrence of AF [13].

We demonstrate, for the first time, a lower success rate of both immediate cardioversion and subsequent maintenance of sinus rhythm in patients with T2DM. Indeed, further regression analysis confirmed that the diagnoses of T2DM and HbA1c among the patients with T2DM are independent risk factors for DC cardioversion failure. Poorer glycaemic control is a known risk factor for CVD [1] and the risk of developing atrial fibrillation [14]. Furthermore, patients with diabetes and AF are more likely to develop cardioembolic stroke [15], as well as having increased morbidity and mortality [16]. It is therefore postulated that limiting the onset of AF and reversion to sinus rhythm may result in improved outcomes [14], although more conclusive evidence for this is required. Improved glycaemic control per se was not shown to impact on the incidence of atrial fibrillation in the recent ACCORD study [16]. A recent study has shown that a higher HbA1c is associated with an increased risk of recurrence of atrial tachyarrhythmia in patients with T2DM undergoing catheter ablation [17]. However, the impact of T2DM and especially the impact of hyperglycaemia on the success of DCCV have not been described to date.

The presence of other microvascular and macrovascular complications may directly influence the development and chronicity of atrial fibrillation in people with diabetes [18]. Indeed, the prevalence of AF was shown to be greater in patients with diabetic autonomic neuropathy compared with patients who have diabetes but without neuropathy [19]. Importantly, cardiac autonomic neuropathy can influence the onset of AF as well as prognosis following DCCV [20]. After adjustment for preexisting macrovascular and microvascular disease, HbA1c remained an independent adverse risk factor for the success of DCCV. Recurrent AF has a low success rate of cardioversion which is secondary to atrial remodeling during AF [21]. This in itself was found to be an independent risk factor for unsuccessful treatment of AF in patients with T2DM.

It is well known that patients treated with digoxin have a lower chance of spontaneous reversions to sinus rhythm, as well as successful DCCV [22]. In this study we show a similar result, particularly in patients with T2DM. In this group it was found to be an independent risk factor influencing the immediate success as well as maintenance of sinus rhythm

at follow-up. Why digoxin use may influence more adverse outcomes is not clear, but altered myocardial calcium homeostasis in patients with T2DM may be a possible explanation [23].

Statins have also been shown to have a favourable impact on attaining and maintaining sinus rhythm in patients with AF [13]. However, we found that concomitant statin usage had a favourable impact on the whole group, but not among the patients with T2DM. This may be due to the myocardial electrophysiological effect of statins which could be mediated by a reduction in CRP [24], interleukins [25], catecholamines [26], and altered cell membrane properties [27], which may well be further attenuated in patients with T2DM.

Structural cardiac alteration with increased LA size [28] in particular is known to influence success in maintaining sinus rhythm in patients undergoing DC cardioversion for AF. Thus AF per se is thought to lead to left-atrial remodeling [28]. We confirm this association in patients with and without T2DM. The other major haemodynamic consequence of persistent AF is its impact on LV systolic function, which can improve after reversion to sinus rhythm [29]. The main mechanism for such a reduction may be the tachycardia-induced LV systolic dysfunction, due to reduced myocardial calcium secondary to shortened diastole [30].

The present study is the largest case-control study identifying a range of risk factors, determining immediate and longer-term outcomes following DC cardioversion in patients with T2DM. A limitation of this study is that the technique for cardioversion used was monophasic DCCV whilst current clinical guidelines advocate the use of biphasic DCCV due to superior efficacy and fewer energy requirements [6]. The success rate for DCCV at the first visit in our study as aforementioned was similar to previous reports [12] and interestingly for patients without T2DM the proportion of those who attained sinus rhythm was 84% which is very similar to the results in a previous relatively large study in which the efficacy of monophasic DCCV was found to be inferior to biphasic DCCV (84% versus 95% success rate) [31]. In that study, out of the 229 patients who had monophasic DCCV for treatment of AF, only 9 had a history of diabetes [31]. Another limitation of this study was that the duration of AF precardioversion was significantly longer in the group of patients with T2DM compared to those without T2DM. Duration of AF is known to be a risk factor for inability to achieve sinus rhythm on first attempt as well as recurrence of AF at follow-up using DCCV treatment [12]. Duration of AF was therefore adjusted in the regression model when determining independent risk factors for both immediate success and relapse. Ventricular rate prior to DCCV was not assessed as a significant proportion of patients were on rate limiting medications. The prevalence of rate limiting medication use, however, did not differ between groups with and without T2DM. We acknowledge that due to the retrospective design of this study certain relevant information such as body mass index was not readily available to us and would have added to the data interpretation. Despite consideration of all of these factors, we believe this study provides important insights into the potential basis for poorer outcomes in patients with T2DM undergoing DCCV.

5. Conclusion

The presence of T2DM and the degree of hyperglycaemia as represented by HbA1c are independent risk factors for immediate and medium-term failure of DCCV in patients with AF.

Additional Points

Key Messages. (i) This study shows that type 2 diabetes negatively impacts on the success of direct current cardioversion (DCCV) for atrial fibrillation (AF). (ii) The presence of T2DM is an independent risk factor for the relapse of AF after treatment. (iii) We report that in patients with type 2 diabetes glycated haemoglobin is a predictor for immediate DCCV failure in AF.

Conflicts of Interest

Handrean Soran received research grants from Synageva, Pfizer, Amgen, and MSD and honoraria from Sanofi, Synageva, BMS, Lilly, AstraZeneca, Pfizer, Takeda, AMGEN, and MSD. Naveed Younis received research grants from Sanofi and honoraria from Jansen, Astra-Zeneca, Lilly, and Novo-Nordisk. Moulinath Banerjee, Safwaan Adam, Shakawan M. Ismaeel, Jan Hoong Ho, Akheel A. Syed, and Rayaz A. Malik have no conflicts of interest to declare.

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Review Article

Coronary Atherosclerosis Assessment by Coronary CT Angiography in Asymptomatic Diabetic Population: A Critical Systematic Review of the Literature and Future Perspectives

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The prognostic impact of diabetes mellitus (DM) on cardiovascular outcomes is well known. As a consequence of previous studies showing the high incidence of coronary artery disease (CAD) in diabetic patients and the relatively poor outcome compared to nondiabetic populations, DM is considered as CAD equivalent which means that diabetic patients are labeled as asymptomatic individuals at high cardiovascular risk. Lessons learned from the analysis of prognostic studies over the past decade have challenged this dogma and now support the idea that diabetic population is not uniformly distributed in the highest risk box. Detecting CAD in asymptomatic high risk individuals is controversial and, what is more, in patients with diabetes is challenging, and that is why the reliability of traditional cardiac stress tests for detecting myocardial ischemia is limited. Cardiac computed tomography angiography (CCTA) represents an emerging noninvasive technique able to explore the atherosclerotic involvement of the coronary arteries and, thus, to distinguish different risk categories tailoring this evaluation on each patient. The aim of the review is to provide a wide overview on the clinical meaning of CCTA in this field and to integrate the anatomical information with a reliable therapeutic approach.

1. Introduction

Diabetes mellitus (DM) is a major public health problem, the incidence of which seems to be drastically increased and will grow in the next years [1, 2]. Many studies in the literature showed a clear correlation between DM and risk of coronary heart disease (CAD). [3–5]. Moreover, compared with matched nondiabetic individuals, patients with diabetes has a higher prevalence, extent, and severity of CAD [6]. On the basis of these considerations and on the beneficial removal of the risk factors on progression of atherosclerotic disease, early detection of diabetic patients at increased risk of adverse cardiac events is crucial.

Coronary computed tomography angiography (CCTA) is an emerging noninvasive technique for the evaluation of coronary stenosis and for the characterization of the atherosclerotic plaques [7–9]. However, although the diagnostic accuracy and prognostic value of CCTA have been largely proved in symptomatic low-intermediate patients [10–13], its role in the asymptomatic and diabetic individuals is still widely debated [14, 15]. The American Diabetes Association and American Heart Association recently issued a

joint statement that urges the identification of asymptomatic patients with subclinical CAD in whom more aggressive lifestyle or treatment changes would allow prevention of progression of the disease and reduce future clinical events [16] In the light of this, the objective of our review is to assess the rationale and effectiveness of CAD screening in asymptomatic diabetic patients by CCTA, providing future perspectives on potentiality of this emerging noninvasive imaging technique.

2. Diabetes Mellitus as Coronary Risk Equivalent: An Unsolved Matter

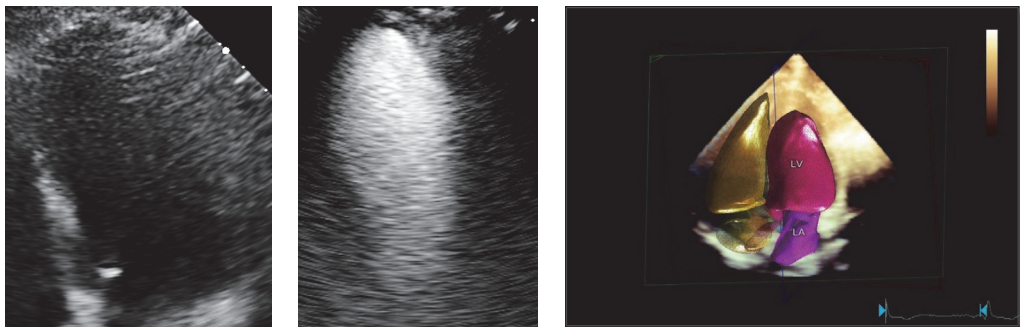
CAD represents the main cause of mortality and morbidity in patients affected by DM, which was considered as a "coronary risk" equivalent [17, 18]. The validated correlation between DM and increased risk of CAD sparked a vivacious debate in the scientific community about the appropriateness of considering the diabetics as patients affected by CAD by default. The consideration of the DM as a "coronary equivalent," however, has a remarkable role because it implies a very aggressive treatment with significant healthcare costs, possible lack of patient's compliance, and risk of adverse effects.

In order to weigh up the risk of CAD in the diabetic population compared to nondiabetic population, Haffner et al. [19] compared the incidence of AMI in 1373 diabetic patients and 1059 nondiabetic subjects in the Finnish population and followed them up for 7 years. The study showed that previous AMI had a substantial role in determination of second AMI, stroke, and cardiovascular death. AMI incidence in nondiabetic population was 18.8% in the population with prior AMI and, conversely, 3.5% in nondiabetic population without prior AMI. In parallel, in diabetes group, the incidence of AMI was 45.0% in the population with prior AMI and 20.2% in the population without prior AMI. On the other hand, nondiabetic patients without previous AMI showed better survival. The substantial novelty of the study is finding a similar incidence of cardiovascular events in the group of 890 patients with DM without prior AMI and in the group of 69 patients without DM but with prior AMI, in a follow-up of 7 years. This correlation is unchanged even after adjustment for demographic variables (age and gender) and other cardiovascular risk factors (smoking, hypertension, lipid profile). On the basis of these results, the authors affirmed the negative impact of DM on coronary perfusion because diabetic patients with no known history of CAD presented the same risk of cardiovascular death as patients without DM but with prior AMI. For this reason, Haffner considered DM as an equivalent of CAD, implying an increase of 20% in the 10-year cardiovascular risk of adverse events. This result suggests and encourages the treatment of all diabetic patients, as if they were really affected by known CAD [20]. Although recent studies confirmed and supported the consideration of "coronary risk equivalency" [21, 22], other bodies of evidence seem to reconsider this assumption, suggesting the identification of different classes of risk [23, 24]. A meta-analysis published in 2009 [25] compared the total risk of coronary events in diabetic patients without previous AMI and nondiabetic patients with previous AMI. This meta-analysis evaluated 13 studies including 45.108 patients with a mean follow-up of 13.4 years and a mean age of the enrolled subjects between 25 and 84 years. 2603 CHD events were found in diabetic population with no previous AMI; on the other hand 3927 events were recorded in the nondiabetic population with prior AMI. This work showed that diabetic patients without previous AMI presented a 43% lower risk of developing coronary events compared with nondiabetic patients with prior AMI (summary odds ratio 0.56, 95% confidence interval 0.53–0.60). This result suggests that, although DM is an important risk factor for the development of cardiovascular adverse events, it cannot be considered as a "coronary risk equivalent." Recently, Rana et al. [26] have studied a large cohort of 1.586.081 adults, admitted to the Kaiser Permanente Northern California healthcare system, aged between 30 and 90 years with a 10-year follow-up. The study compared the risk of adverse cardiac events in the population divided into 4 groups according to the presence of DM and coronary heart disease (CHD). The study confirmed that the sole presence of prior CHD is associated with an almost twofold increased risk of CHD, compared to the presence of sole DM (12.2 versus 22.5 per 1000 person-years), suggesting that the DM is an additional risk factor rather than a trigger in the progression of CAD. Remarkably, only when diabetes was present for more than 10 years, the risk of future CHD for patients with diabetes was similar to that for those with previous CHD. Of note is that, although the Adult Treatment Panel (ATP) III guidelines in 2001 recommended lifestyle and therapeutic primary prevention in diabetics [20], subsequent ACC/AHA American guidelines on the individual risk assessment reduced the DM role in the progression of atherosclerotic disease, on the basis of these new scientific bodies of evidence in the literature [27]. Therefore, there is no scientific evidence to support an aggressive therapeutic strategy with statins and aspirin in all patients with DM, but only in diabetic patients at high risk, in order to reduce cardiovascular mortality. On the basis of this important meta-analysis, it is crucial and essential to identify diabetic patients at high risk of adverse coronary events, worthy of an adequate aggressive therapy with statins and aspirin.

3. Standard Diagnostic Approach to Asymptomatic Diabetic Patient

In view of the high prevalence of CAD and the nonnegligible autopsy rates of silent coronary ischemia in diabetic patients due to prevalent neuropathy [28], noninvasive stress imaging could be useful in the prognostic stratification of asymptomatic diabetic patients in order to minimize vascular consequences of chronic hyperglycemia and optimize therapeutic approach.

Detecting CAD in patients with diabetes is challenging [29]. The involvement of small vessels due to metabolic abnormalities and the diffuse nature of the disease limit the reliability of cardiac stress tests for detecting myocardial ischemia [17], further worsened by the comorbidities (Figure 1). In addition, the silent fashion of CAD due to the high threshold for pain reduces the sensitivity of clinical risk assessment [30].



(a) (b) (c)

Figure 1: Dobutamine stress echocardiography in patient affected by diabetes mellitus (DM) with chronic pulmonary obstructive disease (COPD) and poor acoustic window. (a) Apical four-chamber view of left ventricle. (b) The use of ultrasound contrast agent definitively allows an acceptable imaging quality of the endocardial border. (c) 3D heart model is imaged. It represents a chamber quantification using a model-based segmentation algorithm for achieving a more accurate functional assessment.

Exercise electrocardiography (EKG) is the most used noninvasive technique for the diagnostic and prognostic evaluation of nondiabetic patients with known or suspected CAD. Unfortunately, the accuracy of exercise EKG is reduced in the diabetic population. The poor response in terms of pressure and heart rate increasing during exercise, the high incidence of silent myocardial ischemia and microvascular disease, the alterations of impulse conduction due to visceral neuropathy, the presence of baseline ST-segment abnormalities, left ventricular hypertrophy, and the impaired exercise capacity due to peripheral vascular disease limited the diagnostic and prognostic value of exercise EKG. Given the limited accuracy of exercise EKG stress in diabetic patients, myocardial stress imaging tests have been proposed [31] also thanks to their ability to identify changes in regional contractility depending on the location and size of the ischemic area. In asymptomatic diabetic patients, the sensitivity and specificity of stress echocardiography in the diagnosis of CAD are reported to be 81% and 85%, respectively [32].

Stress echocardiography allows identifying, in the absence of signs of stress ischemia, patients at low risk of developing adverse cardiac events [33, 34]. Two studies conducted on diabetic sample revealed a small number of false negatives in the identification of CAD, sustaining good efficacy of stress echocardiography in identifying low risk diabetic patients to develop cardiac events [35, 36]. Few years later, Kamalesh et al. [37] studied the incidence of adverse cardiac events in a diabetic and nondiabetic population with absence of signs of inducible ischemia as assessed by stress echocardiography and followed them up for 25 ± 7 months. The study showed that diabetic patients, compared to nondiabetic ones, had a higher incidence of cardiac events (19% versus 9.7%, $p = 0.03$), worse event-free survival ($p = 0.03$), and a greater number of nonfatal MI events (6.7% versus 1.4%, $p < 0.05$). The study revealed also that the history of CAD was the only predictor of adverse cardiac events ($R = 0.18, p < 0.05$). On the basis of these results, Kamalesh concluded that diabetic patients with negative stress echocardiogram have more risk for adverse cardiac events compared to nondiabetic patients. The cause of this substantial difference could be explained by the greater tendency of diabetic patients to have distal CAD, slightly detectable by stress echocardiography. Moreover, diabetic patient presents an alteration of the coagulation pattern, intense platelet activity, and reduced fibrinolysis which, together with the recognized autonomic dysfunction, most frequently predispose the patient to coronary occlusion [38]. On the same line, Cortigiani et al. [39] showed that a negative, nonischemic stress test in the diabetic population, particularly in the subset of patients aged >65 years, is associated with an increased risk of developing adverse cardiac events when compared to nondiabetic subjects of the same age. Confirming these results, other studies showed an annual incidence of adverse cardiac events in diabetic patients with normal stress test equal to 3–6%, about twice that in nondiabetic patients with normal stress test [40, 41]. On the other hand, some large studies assessed the prognostic value of single-photon emission computed tomography imaging in patients with DM and, importantly, also in this scenario the event rate was higher compared with the control population, even in presence of a normal scan [29, 42].

On the basis of these considerations, although exercise stress testing and myocardial perfusion imaging remain important techniques for risk assessment and prognosis of CAD in asymptomatic diabetic patients, presence of confounding factors, such as autonomic dysfunction, multivessel disease, EKG abnormalities and interpretative difficulties, peripheral artery disease, and the need for polypharmacotherapy, could compromise the diagnostic efficacy explaining why their role remains controversial [43].

4. Calcium Scoring

Coronary artery calcium score (CACS) is widely considered a marker of subclinical atherosclerosis, validated in asymptomatic patients [47]. Extent of CACS, in fact, well correlates with the vascular atherosclerotic involvement and the probability of adverse cardiac events in the general population [48–50]. Although the latest European guidelines on cardiovascular prevention [51] suggested evaluation of

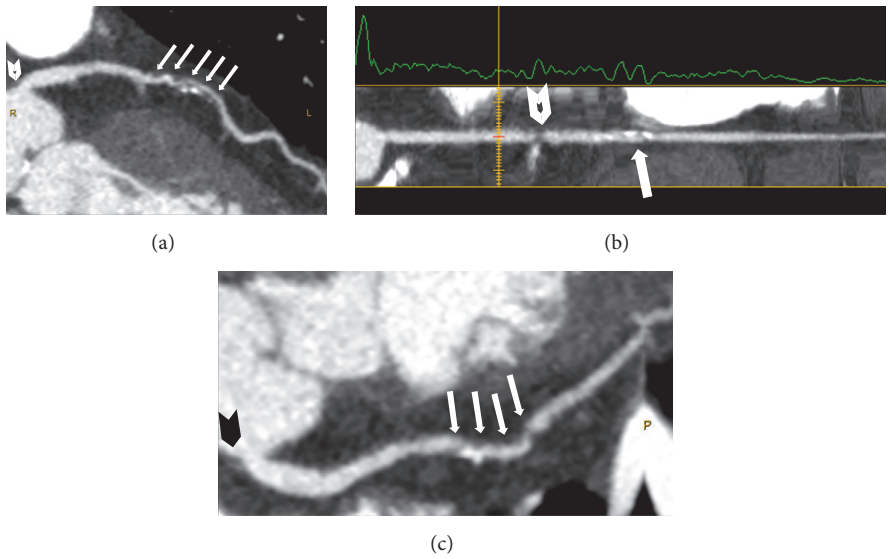


Figure 2: Computed tomography coronary angiography (CTCA) showing three-vessel disease. (a) Left anterior descending (LAD) artery. White arrows indicate a long, mixed, and severely obstructive plaque at proximal-middle LAD. White arrowhead shows a noncalcified, nonseverely obstructive plaque at the origin of left main artery (LM). (b) Right coronary artery (RCA). White arrow indicates a mixed and nonseverely obstructive plaque at middle RCA. White arrowhead shows a noncalcified, severely obstructive plaque at proximal RCA. (c) Left circumflex artery (LCx). White arrows indicate a long, mixed, and severely obstructive plaque at proximal-middle LCx. Black arrowhead shows a noncalcified, nonseverely obstructive plaque at the origin of LCx.

the CACS only in diabetic patients with high or very high cardiovascular risk (score > 5% and score > 10%), the latest American guidelines for risk stratification in patients with CAD recommended an "appropriate" use of CACS and CCTA in asymptomatic patients with high global risk [52].

Type 2 DM patients have higher values of CACS when compared with the general population [53]. The mechanisms responsible for the extensive intracoronary calcium accumulation in diabetic patients are multifactorial and not completely understood. Previous studies revealed that the increased production of advanced glycation end-products induces the overexpression of genes and enzymes involved in active calcification of the coronary plaque [54]. Coronary artery calcium scoring (CACS) has been proposed as a first-line test for CAD in patients with diabetes [55] since it was widely demonstrated that it has higher capability with respect to conventional cardiovascular risk factors for predicting silent myocardial ischemia and short-term outcome [56]. Numerous studies showed that higher values of CACS in diabetic patients with metabolic syndrome are closely associated with increased prevalence of ischemia, adverse cardiac events, AMI, and mortality [57–60].

Notwithstanding, a significant percentage of patients with DM have very low or zero CACS, with a better long-term prognosis, revealing that DM is not an equivalent of coronary risk. Raggi et al. documented a high proportion of asymptomatic patients with DM (39%) with CACS < 10 [61]. In this study the authors confirmed a significant correlation between CACS and DM ($p = 0.00001$), indicating that each increase of CACS correlates with an increase in mortality in diabetic and nondiabetic patients. However, diabetic patients without known CAD showed similar survival to patients without DM and intracoronary calcium (98.8% and 99.4%, resp., p: 0.5). The results of other studies show the same trend [62, 63].

5. Coronary Computed Tomography Angiography (CCTA)

Recently, CCTA has emerged as a reliable noninvasive imaging tool for the identification of CAD [64–68]. Since is first steps, the technique has been characterized by a very high negative predictive value, whereas the positive predictive value has been growing progressively, mainly according to the improvement of many technical aspects [69]. The suboptimal positive predictive value and specificity of CCTA in assessing the coronary stenosis degree are mostly due to the "blooming" artifacts secondary to the presence of wall calcifications. In particular, the coronary arteries in diabetic subject are characteristically "small and calcific," and this explains why the specificity of CCTA in this specific subset of patients may be particularly low (Figure 2). At the same time, the technological innovation has been taking a giant step towards the artifacts reduction by implementing different strategies throughout the process, from the premedication of the patient before scanning to the acquisition and analysis of the images [70–74]. Of note is that a new important and very attractive tool able to evaluate the functional value of a single stenosis, the fractional flow reserve CT (FFR_{CT}), is not influenced by the presence of calcifications and thus is particularly reliable in diabetic population (Figure 3) [75–79]. Among all others, the employment of high definition techniques [80] allows high values of specificity and diagnostic accuracy (close to 90% and 95–98%, resp.).

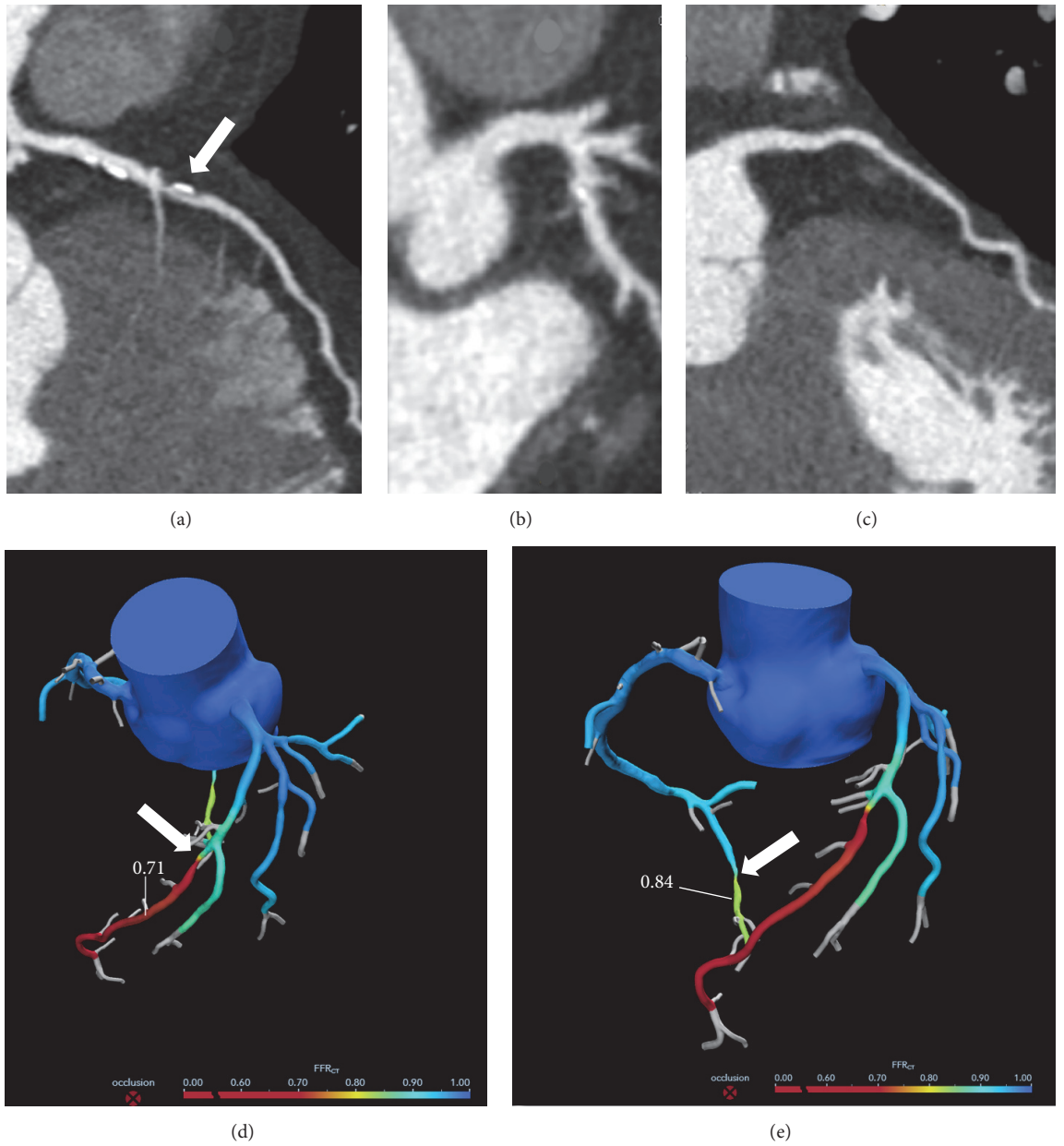


Figure 3: Computed tomography coronary angiography (CTCA) and fractional flow reserve CT (FFR_{CT}). (a) Left anterior descending (LAD) artery. White arrow indicates a mixed, severely obstructive plaque at middle LAD. (b) Right coronary artery (RCA) with a small calcific spot. (c) Left circumflex (LCx) artery does not show atherosclerotic plaques. (d), (e) Graphic representation of FFR_{CT} calculation. (d) Following LAD stenosis (white arrow), the value is 0.71. (e) Following RCA stenosis (white arrow), the value is 0.84.

Pivotal information is obtained by CCTA, specifically that obstructive and nonobstructive CAD are characterized by a higher prevalence in the diabetic population compared to normoglycemic patients and that a different plaque composition does exist [81–83]. Table 1 shows as a whole that among patients with DM, nonobstructive and obstructive CAD according to CCTA are associated with higher rates of all-cause mortality and major adverse cardiovascular events at follow-up, and this risk is significantly higher than that in nondiabetic subjects. Despite this, current European guidelines do not advise coronary CTA for risk assessment and suggest other noninvasive testing methods (nuclear imaging, echocardiography, and carotid ultrasound) in high risk diabetic patients [84]. Conversely, the latest American guidelines for detection and risk assessment of stable CAD state that calcium scoring and coronary CTA use "may be appropriate" in asymptomatic patients with high global risk [52, 85].

The fulcrum of noninvasive coronary assessment in diabetic population consists in its prognostic value. Numerous efforts have been made so far in order to add useful information on this debated topic.

Table 1: Randomized studies that investigated the prognostic power of CCTA in asymptomatic or stable patients with diabetic mellitus versus nondiabetics.

Authors/journal	Diabetics	Nondiabetics	Follow-up	Events in diabetics	Events in nondiabetics	Characteristic of CAD at CCTA	Univariate analysis in diabetics (HR)	Multivariate analysis in diabetics (HR)	Univariate analysis in nondiabetics (HR)	Multivariate analysis in nondiabetics (HR)
Van Werkhoven et al./Radiology 2010 [44]	$n = 313$	$n = 303$	20 ± 5.4 months	Total cardiac events (88)	Total cardiac events (45) $p < 0.001$	Obstructive	6.57 ($p < 0.001$)		16.29 ($p < 0.001$)	21.64 ($p < 0.001$)
Rana et al./Diabetes Care 2012 [6]	$n = 3370$	$n = 6740$	26 months	Death $n = 108$ (3.2%)	Death $n = 115$ (1.7%)	Nonobstructive		5.25		3.12 ($p < 0.01$)
						(1) Vessel disease		6.39		5.56 ($p < 0.01$)
						(2) Vessel disease		12.33		7.87 ($p < 0.01$)
						(3) Vessel disease		13.25		9.25 ($p < 0.01$)
Nadjiriet et al./Int J Cardiovasc Imag 2015 [45]	$n = 108$	$n = 1379$	66 ± 12.2 months	Cardiac events $N = 10$ (annual cardiac event rate 1.74%)	Cardiac events $N = 48$ (annual cardiac event rate 0.64%)	Number of lesions per patient (SIS)		3.0 ($p = 0.047$)		
						Segment stenosis score (SSS)		4.5 ($p = 0.025$)		
						Number of lesions per patient (SIS)				1.31 ($p = 0.076$)
						Segment stenosis score (SSS)				1.3 ($p = 0.062$)
Blanke et al./Jacc CI 2016 [46]	$n = 1823$	$n = 1823$	60 months	Death $n = 246$ (13.5%)	Death $n = 136$ (7.5%)	*RR for all-cause mortality in diabetics compared with propensity matched nondiabetics stratified according to extent/severity of CAD*				
						Nonobstructive		2.09 ($p < 0.001$)		
						Obstructive		1.95 ($p < 0.001$)		
						(1) Vessel disease		1.48 ($p = 0.08$)		
						(2) Vessel disease		2.45 ($p = 0.003$)		
						(3) Vessel disease		2.31 ($p = 0.002$)		

CAD: coronary artery disease; CCTA: coronary computed tomography angiography; HR: hazard ratio; RR: relative risk.

Min et al. [86] evaluated the prognostic value of CCTA in a population of 400 asymptomatic diabetic patients without known history of CAD. This study showed that, after adjustment for CAD risk factors, the maximum stenosis, the number of coronary arteries involved, and the segment stenosis score are associated with increased risk of developing adverse cardiac events and had incremental power for predicting cardiac events over conventional risk factors. Moreover, the study revealed that CCTA confers incremental risk prediction, discrimination, and reclassification over CACS. Based on these results, CCTA seems to be very useful in risk stratification of asymptomatic diabetic patients at higher risk of developing adverse cardiac events. Halon et al. [87] examined the added value of CCTA over clinical risk scores of United Kingdom Prospective Diabetes Study (UKPDS) and coronary artery calcium in a population based cohort of 630 asymptomatic type 2 diabetics with no history of CAD assessed for coronary heart disease related events over 6.6 ± 0.6 years. Discrimination of all events was improved by addition of total plaque burden to the clinical risk and CACS combined and further improved by addition of an angiographic score.

Van Werkhoven et al. [44] confirmed the usefulness of CCTA in prognostic stratification of diabetic patients ($N = 313$) with known or suspected CAD compared to nondiabetic patients ($N = 303$). Authors found that DM ($p < 0.001$) and evidence of obstructive CAD (>50% coronary stenosis) ($p < 0.001$) were independent predictors of outcome. In particular and similarly to other bodies of evidence [88, 89], the presence of obstructive CAD is an important predictor of survival both in diabetic patients and in nondiabetic patients. Conversely, absence of atherosclerosis in CCTA is associated with excellent (100%) disease-free survival at a mean follow-up of 20 ± 5.4 months, confirming the known high predictive value of CT both in diabetic and in nondiabetic patients [90, 91].

Furthermore, the study conducted by Kim et al. [92] demonstrated that the duration of DM is significantly associated with the extent and the severity of CAD. Patients with a longer history of DM had higher levels of CACS, atheroma burden obstructive score, segment involvement score, and segment stenosis score ($p < 0.001$ for all). In addition, the severity of coronary stenosis clearly increases the incidence of adverse cardiac events, independently of other cardiovascular risk factors. On the basis of these considerations, authors suggest the introduction of CCTA screening in all patients with a history of DM > 10 years.

On the contrary, the study of Muhlestein et al. revealed that the use of CCTA as screening of asymptomatic diabetic patients did not reduce the incidence of mortality from all causes and nonfatal myocardial infarction. However, the value of this result could be resized taking into consideration the low incidence of adverse cardiac events in the study which reduces the statistical difference between the two groups [93]. Other than being unpowered, the study was biased by the fact that adequate care targets for risk factor reduction in most of patients assigned to receive aggressive therapy in CCTA group were not achieved. Moreover, the control group without CTA scanning also received good preventive medical treatment so that differences in therapy between the screened and nonscreened groups were subtle.

Recently, Kang et al. confirmed the prognostic value in long term of CCTA in a population of asymptomatic diabetics [94]. This study analyzed clinical outcome of 591 asymptomatic patients with type 2 DM undergoing CCTA showing that the survival free of cardiac events was 99.3 ± 0.7% in patients with normal coronary arteries, 96.7 ± 1.2% in those with nonobstructive CAD, and 86.2 ± 3.0% in those with obstructive CAD (log-rank $p < 0.001$). The present study confirmed that asymptomatic diabetic patients with normal coronary arteries or with nonobstructive CAD have an excellent clinical outcome even after five years, conversely to patients with obstructive CAD. An overview is given by a recent meta-analysis based on eight studies with a total of 6225 participants (56% male with average age of 61 years) and a mean follow-up of 20 to 66 months that evaluated the prognostic efficacy of CCTA in diabetic patients [95]. This meta-analysis concluded that CCTA is critical in identifying diabetic patients at high risk of CAD to be assigned to an aggressive modification of risk factors, glycemic control, and optimized medical therapy.

6. Therapeutic Perspectives

At this point it is not incorrect to say that CCTA is able to distinguish between high and low risk diabetics patients, unveiling the presence of severe CAD. At the same time, CCTA can detail anatomic information of CAD features providing incremental power in the context of primary prevention of acute cardiac events [96]. The ability of this technique in revealing some vulnerability features of coronary plaque is known, including positive remodelling, presence of large plaque burden, and spotty calcification which increase the probability of plaque rupture and complication. Sometimes the "anatomic" high risk condition coexists in a "systemic" vulnerable context depicted by DM and kindled inflammatory state [97, 98] (Figure 4). A recent study [98] reported that diabetic subjects with increasing circulation levels of interleukins-6 and carotid artery disease had high probability of obstructive CAD and high risk plaques. Notwithstanding, the CV risk of the diabetic population is not uniform, and the vital and decisive pivot of the right identification of high risk subset of diabetics consists in impact on prophylactic therapy. The European Society of Cardiology guidelines recommend the consideration of aspirin use for primary prevention in patients at high risk with DM [17], while the Endocrine Society Clinical Practice guidelines recommend aspirin in patients with DM aged >40 years and whose 10-year CV disease risk is more than 10% [99]. Moreover, the existing risk charts tailored on patients with DM, such as the United Kingdom Prospective Diabetes Study and the Swedish National Diabetes Register [100, 101], need further validation for clinical applicability. Although noninvasive imaging tests demonstrated their value in risk stratification of diabetic subjects [102], no mention is made of the need for incorporating them in the diagnostic flow charts.

In a valuable attempt to correlate traditional CV risk factors with anatomic CAD features, Dimitriu-Leen et al. [103]

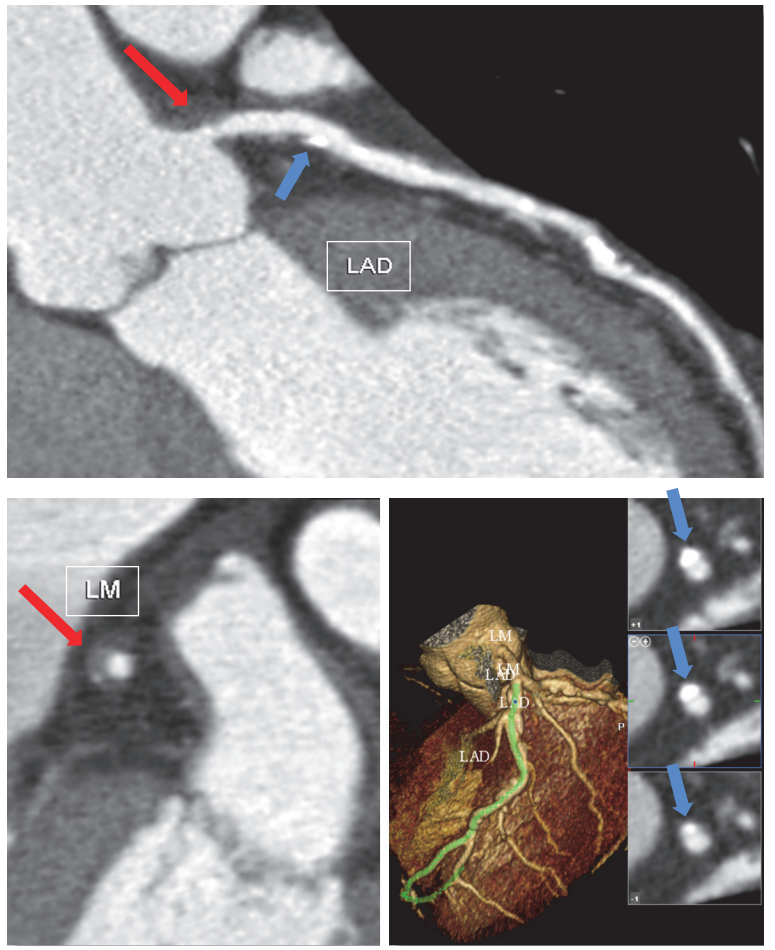


Figure 4: Diabetic patient with carotid disease, active inflammatory pattern, and multivessel coronary artery disease (CAD). The sole coronary artery calcium scoring (CACS) and coronary invasive angiography do not entirely represent the effective risk of the patients (CACS Agatston score 130 and stenosis of 30% of left main artery [LM]). The red arrow represents a very large, remodelled plaque with a little calcific component at the level of LM and thus a "high risk plaque." The blue arrow represents a large, remodelled plaque with a bigger calcific component at the level of left artery descending (LAD). In this case the plaque does not generate any significant stenosis. Overall, these CAD features confer an incremental event risk.

prospectively studied a large asymptomatic diabetic population at high risk. On CCTA, 27% of these patients had no CAD. Considering patients with any CAD (73%), around half had obstructive CAD (more than 50% stenosis). Importantly, the study showed that the number and presence of risk factors were not associated with a higher frequency of CAD, except for hypertension. As a consequence, the authors underlined that CCTA could be pivotal in identifying which patients will benefit most from prophylactic prevention with aspirin. In this regard, it is necessary to keep in mind that aspirin is only useful if coronary atherosclerosis is present [104]. Notably, screening patients according to their CACS instead of exploring CAD on CCTA would result in undertreatment of 9% (diabetics with obstructive CAD) to 36% (diabetics with any CAD) of patients at high risk with DM who may benefit from therapy and this is in line with what other authors have shown [105]. The importance of well defining the high risk diabetic subjects worthy of prophylactic aspirin therapy derives from the evidence that the trials aimed at establishing its beneficial effect have been controversial and, particularly, 2 of those have failed in demonstrating significant reductions in CV events [106, 107]. Taylor et al. [108] in their analysis revealed the poor utility of statins use in diabetic population. This result, apparently paradoxical, highlights that diabetic patient should not necessarily be considered as a "coronaric" patient to be subjected to intensive medical therapy. Diabetic patients, in fact, in the presence of CACS < 10, have a brilliant prognosis, comparable with nondiabetic patients. Moreover, a substudy of the "coronary CT angiography evaluation for clinical outcomes: an international multicenter (CONFIRM) international registry" demonstrated in 4,706 patients with nonobstructive (less than 50% stenosis) CAD that prophylactic aspirin use was not associated with an improvement in all-cause mortality.

Although these bodies of evidence aim to demonstrate a rationale employment of prophylactic therapy, it seems

clear that more comprehensive prospective studies, including inflammatory biomarker and polyvasculopathy assessment together with preventive treatment strategies, are warranted.

7. Conclusions

There is marked heterogeneity of risk among diabetic patients which has recently gained by scientific community. Clinical risk assessment, standard noninvasive imaging techniques, and CACS alone lack very accurate and tailored risk stratification at single level patient. Coronary computed tomography angiography represents a new technique able to detail CAD features providing diagnostic and prognostic information on asymptomatic type 2 diabetics. The direct consequences of this are that a significant proportion with no or very little coronary plaque are at negligible risk and others with more extensive plaque at considerably higher risk for an acute coronary event. Moreover, the prognostic prediction is refined with the consideration of plaque composition and with the assessment of inflammatory/polyvascular systemic involvement. In diabetics at low risk, the intensity of preventive medical therapy and frequency of follow-up may be reduced, particularly when there is intolerance to aspirin and high doses of statins or other prophylactic therapies. Afterwards, a stepwise approach of screening on the basis of cardiovascular risk factors and global clinical risk would allow characterization of a higher-risk group in which CACS followed by CTA is able to further risk stratification. In the setting of patients with more than 10 years of disease, a direct anatomical imaging strategy may allow the quick and reliable risk stratification of each patient. The identification of significant CAD in the context of a patient with DM could justify an intensive preventive regimen based on aspirin and, accordingly, on clinical conditions, statins, and antihypertensive drugs.

Disclosure

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Conflicts of Interest

The authors declare that they have no relationship with industry or financial associations within the past 2 years which poses conflicts of interest in connection with the submitted article.

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Research Article

Association between Fluorescent Advanced Glycation End-Products and Vascular Complications in Type 2 Diabetic Patients

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Objectives. Diabetes is a major health problem associated with hyperglycemia and chronically increased oxidative stress and enhanced formation of advanced glycation end-products (AGEs). The aim of this study was to determine whether oxidative plasma biomarkers in diabetic patients could be evidenced and associated with vascular complications. *Methods*. Oxidative stress biomarkers such as thiols, ischemia-modified albumin (IMA), glycated albumin (GA), fructosamine, and AGEs were measured in 75 patients with poorly controlled type 2 diabetes (HbA1c > 7.5%) with (44) or without (31) vascular disease and in 31 nondiabetic controls. *Results*. Most biomarkers of oxidation and glycation were significantly increased in diabetic patients in comparison with nondiabetics. Fructosamines, GA, IMA, and AGEs were positively correlated and levels of fluorescent AGEs were significantly increased in the plasma from patients presenting vascular complication. *Conclusions*. These results bring new evidence for the potential interest of glycated albumin, oxidative stress, and glycoxidation parameters in the monitoring of type 2 diabetic patients. Furthermore, it emphasizes fluorescent AGEs as a putative indicator for vascular event prediction in diabetic patients.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disease which has reached pandemic proportions and is considered as one of the world's most important causes of healthcare expenditure, disability, and mortality. Enhanced morbidity in diabetes is mainly associated with micro- and macrovascular complications [1]. Indeed, T2DM could double the risk of developing cardiovascular diseases (CVD) including stroke and myocardial infarction, which represent the leading cause of mortality in western and developing countries [2]. In Réunion Island, a French overseas territory, diabetes prevalence is particularly high (>20% of adult people) and is associated with a higher vascular disease-related mortality (×2) when compared to mainland France [3].

Glycation is clearly identified as a deleterious phenomenon in diabetic complications and the association with oxidative stress seems to be deleterious too [4, 5]. One of the underlying features of hyperglycaemia is the excessive nonenzymatic glycation of the two main circulating proteins: haemoglobin and albumin. This chemical process consists in a complex cascade of reactions between glucose or derivatives with proteins, yielding a heterogeneous class of compounds termed advanced glycation end-products (AGE) [6]. Glycation process, in conjunction with oxidative stress named "glycoxidation" can cause structural and functional impairments of plasma proteins in particular albumin [7–9] and was involved in pathophysiological mechanism of vascular diseases in T2DM [10, 11]. Many clinical studies have suggested glycated albumin (GA) and ischemia-modified

albumin (IMA) as additional and/or alternative plasma biomarkers of poor glycemic control, redox state, and glycation levels in diabetic subjects [12–14]. Similarly, the redox state of plasma thiols could also be used as a marker for several pathologies such as early atherosclerosis and liver disease [15, 16]. Considered as an important intracellular redox regulator with a potential role in stress signalling, plasma thiols levels could be suggested as an oxidative stress biomarker [17].

Identification of novel vascular risk biomarkers as potential indicators for CVD and stroke prevention and intervention in T2DM remains highly warranted. The increase of plasma levels of glycoxidation and oxidative stress markers could be associated with an increase in risk of developing vascular diseases [18, 19]. Both glycation and oxidative stress may produce biomarkers (AGE and IMA for instance) that could be used to predict vascular disease [10, 20].

The aim of this study was to determine whether oxidative plasma biomarkers in diabetic patients could be evidenced and associated with vascular complications related to diabetic micro- and macroangiopathy.

2. Materials and Methods

2.1. Subjects. The Alb-Ox ERMIES is an ancillary, pilot study of ERMIES (NCT01425866), in which we performed a case/case/control analysis of 106 patients. The diabetic cases considered here are from the ERMIES study (structured self-management education maintained over two years in insufficiently controlled type 2 diabetic patients) whereas controls come from patient samples of biochemistry unit of local hospital (Saint-Denis, La Réunion). Blood was sampled on EDTA tubes (BD Vacutainer).

Diabetic subjects from ERMIES are T2DM patients from La Réunion aged over 18 years with high HbA1c levels (>7.5% for more than three months) enrolled between October 2011 and April 2014 (D, $n = 75$) [21]. They were treated with OHA and/or GLP-1 (oral hypoglycaemic agents/Glucagon-like peptide 1) analogue and/or insulin therapy for at least one year and they followed a stable drug treatment regimen for at least 3 months. All patients with initial severe complications including ischemic or proliferative diabetic retinopathy, severe chronic renal failure, active coronary artery insufficiency, diabetic foot lesion, or cancer were excluded. In the control group (ND, $n = 31$), patients were over 18 years old and had no symptom of uncontrolled diabetes with low HbA1c levels (<6.0%). None of the control subjects was under antidiabetic medication. All eligible subjects provided information on the history of their pathology (previous vascular events, medication, etc.) and underwent a medical examination aimed at assessing their micro- or macroangiopathic complications. All biological data related to the Alb-Ox ERMIES study are given in Table 1.

The pool of diabetic patients was divided into two groups according to the degree of vascular disease assessed in the ERMIES study entry (medical history and clinical review). The main vascular complications (diabetic micro- and/or macroangiopathy) listed in this study are nephropathy, retinopathy, peripheral neuropathy, diabetic foot for microangiopathy, acute coronary syndrome, coronary angioplasty, stable angina, and ischemic stroke for macroangiopathy. Vascular complications were evaluated during the open-label lead in phase (OL LI) following a clinical and biological examination. Type 2 diabetes group included $n = 44$ subjects with a history of vascular disease (VD) and $n = 31$ subject without any vascular complication before the study or at the time of the initial enrolment (NVD).

2.2. Determination of HbA1C Levels. Glycated haemoglobin levels were assessed on anticoagulated whole blood stored at 4°C less than 48 hours after collection in EDTA tubes (BD Vacutainer). HbA1c levels were measured by using a capillary electrophoresis method performed on automate Capillary Flex (SEBIA laboratory).

2.3. Biochemical Analysis. Total proteins, albuminemia, fructosamine, triglyceride (TG), total cholesterol (CHOL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), Apolipoprotein A1 (ApoA1), and Apolipoprotein B (ApoB) levels in plasma were determined using a clinical biochemistry automate Cobas C501 analyzer (Roche Diagnostic). Analyses were performed on EDTA-plasma within 6 hours after blood sampling. Total blood and plasma samples were stored at room temperature during these analyses. Then, plasma samples were stored at −80°C before additional biochemical tests.

2.4. Additional Biochemical Characterizations. Thiols group concentration in plasma proteins was measured by Ellman's assay using 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) [22] as previously described [23].

Glycated albumin and AGE levels were quantified in plasma by using a commercially available ELISA kits for detection of human GA and AGEs (Sunred Bio) according to the manufacturer's protocol. The AGE ELISA commercial kit quantifies preferentially nonfluorescent AGEs (including N(ε)-Carboxymethyllysine (CML) or N(ε)-Carboxyethyllysine (CEL)) relative to fluorescent AGEs such as Pentosidine or Vesperlysine [6].

The fluorescence intensity of AGEs in plasma was obtained at 380 nm excitation and 420 nm emission wavelengths using a Horiba FluoroMax®-4 spectrophotometer. The excitation and emission slits were equal to 5 and 10 nm, respectively. All protein samples were prepared at 1.5 mg/mL in 50 mM sodium phosphate buffer at pH 7.4. Fluorescent AGE (Fluo-AGE) (mainly Pentosidine, Vesperlysine, and Crossline) levels were expressed in arbitrary units.

The albumin-cobalt binding (ACB) test reported by Bar-Or et al. was originally designed to detect ischemia-modified albumin (IMA) in patients with myocardial ischemia [12, 24]. This assay based on the reduced binding affinity of human serum albumin for metal ions (Cobalt, Co^{2+}) was applied here on human plasma samples, as previously described [25]. Preparations for the Co (II) albumin binding protocol consist in the addition of 20 μl of samples (0.15 mM) to 15 μl of a 0.2% cobalt chloride solution, followed by vigorous mixing and 15-min incubation at 37°C. Dithiothreitol (DTT,

Table 1: Clinical and biochemical characteristics of the study participants.

	ND	D	
N	$n = 31$	$n = 75$	
Male Sex (%)	$n = 9$ (29.0)	$n = 22$ (29.3)	
With vascular complication	$n = 0$	$n = 44$	
	Average +/− SD	*Average +/− SD*	*p value*
Age	51.2 +/− 17.7	59.1 +/− 10.3	0.0164
BMI		30.2 +/− 5.5	
Total protein (g/L)	69.8 +/− 6.3	70.6 +/− 4.5	0.5201
Albumin (g/L)	40.5 +/− 7.1	42.3 +/− 2.8	<0.0001
Fructosamine (mol/g)	2.67 +/− 0.41	4.78 +/− 0.77	<0.0001
HbA1c (%)	5.3 +/− 0.43	9.1 +/− 1.1	<0.0001
Total cholesterol (mmol/L)	4.4 +/− 0.82	4.14 +/− 1.04	0.2488
HDL-cholesterol (mmol/L)	1.27 +/− 0.44	1.12 +/− 0.29	0.0771
LDL-cholesterol (mmol/L)	2.82 +/− 0.75	2.50 +/− 0.90	0.1079
Triglycerides (mmol) (25–75th percentile)	1.42 (0.99–1.78)	1.26 (0.98–1.66)	0.67
ApoA (mmol/L)	1.42 +/− 0.37	1.30 +/− 0.22	0.0807
ApoB (mmol/L)	0.84 +/− 0.16	0.89 +/− 0.25	0.3291

Study participants are divided into two groups: diabetic (D, $n = 75$) and nondiabetic (ND, $n = 31$) patients.

20 μl of a 1.5 g/l solution) was then added and mixed. After incubation for 2 minutes, 20 μl of 0.9 M NaCl solution was added. The absorbance ($D1$) was read at 470 nm using a microplate reader. The corresponding blank was prepared similarly without DTT and read at the same wavelength ($D0$). The IMA index was expressed as absorbance $\Delta D = D1 - D0$ which represents the residual unbounded Co^{2+} amount and then normalized to plasma albumin concentration according to the following formula:

$$\frac{\text{IMA}}{\text{ALB}} = \Delta D * [\text{ALB}]. \quad (1)$$

In the previous formula, [ALB] corresponds to serum albumin concentration (g/L).

2.5. Statistical Analysis. Means ± SD or standard error to the mean (SEM) were presented for normally distributed variables and medians (25th–75th percentile) for nonnormally distributed variables. Statistical significance was determined using Student's t-test or the Mann–Whitney test. For regression analyses, we log-transformed all biomarker concentrations with skewed distributions. Spearman's correlations were used to assess the relationships between biomarkers. Univariate and multivariate logistic models were considered to assess the association between AGE levels and the presence of microangiopathy after adjustment for potential confounders. The multivariate model was adjusted for cardiovascular risk factors: age, sex, and body mass index. P values < 0.05 were considered statistically significant. Analyses were conducted using STATA 13 software (STATA corp, Tex, USA).

3. Results

The clinical and biochemical characteristics of participants are reported in Table 1. Type 2 diabetes group included 31 obese patients (BMI > 30) including those who presented severe obesity (BMI > 40, $n = 5$). Patients with T2DM had significantly higher levels of albumin, fructosamine, and HbA1c than controls. In contrast, for others biochemical parameters (triglycerides, cholesterol, and apolipoproteins) the differences did not reach statistical significance. As shown in Figure 1, several oxidation and glycation biomarkers were significantly higher in the diabetic group (D) as compared to the control group: IMA, GA, and fructosamine. Conversely, thiols levels were significantly lower in the diabetic group (1.04 ± 0.02) than in the control group (2.47 ± 0.03). Only nonsignificant variations could be observed for glycoxidation biomarkers; Fluo-AGE and AGE levels were, respectively, higher and lower in diabetic patients relative to controls.

As featured in Table 2, different correlations were established between biomarkers of oxidative stress (thiol, IMA), glycation (glycated albumin, fructosamine), and glycoxidation (Fluo-AGE and AGE). Significant correlations were established between these biomarkers: IMA index was found to be positively correlated with fructosamine ($r = 0.430$, $p < 0.0001$), GA ($r = 0.302$, $p < 0.0001$), and with Fluo-AGE ($r = 0.219$, $p < 0.05$) levels. IMA index does not appear to be correlated with AGE levels. As expected, a marked relationship was noticed between fructosamine and GA ($r = 0.492$, $p < 0.0001$) and to a lesser extent between AGE and GA ($r = 0.399$, $p < 0.0001$). However, there was no significant correlation between thiol levels and other markers.

The clinical and biochemical characteristics of diabetic patients according to the history of vascular disease are summarized in Table 3. Forty-four diabetic subjects presented a vascular disease such as microangiopathy ($n = 30$), macroangiopathy ($n = 3$), or both complications ($n = 11$). The comparison between both groups did not show any significant difference in biological parameter levels. As for specific biomarkers of oxidative stress, glycation,

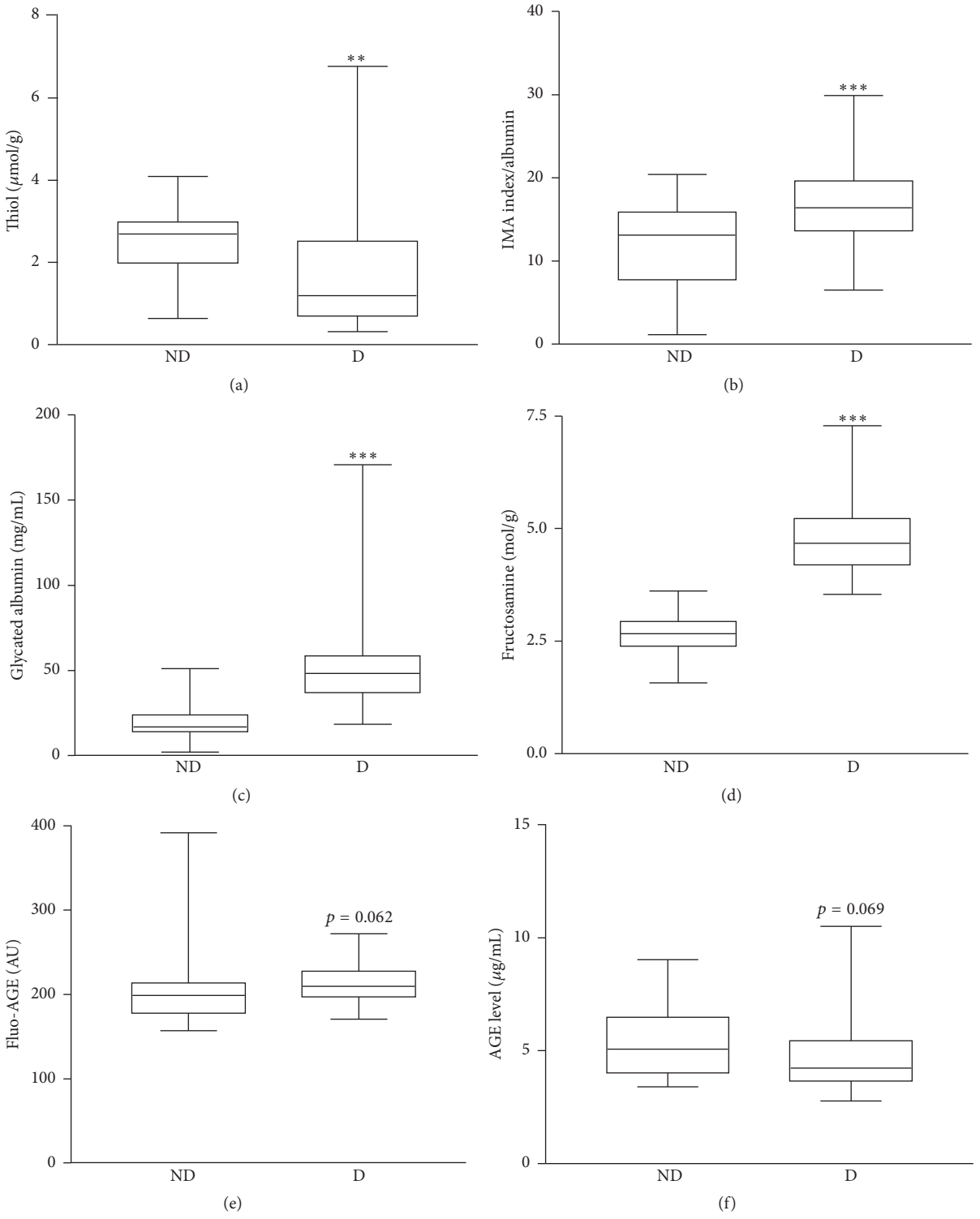

Figure 1: Box-plot distribution of biomarker values in diabetic (D) and nondiabetic (ND) groups. (a) Thiol levels (μmol/g protein); (b) IMA index (AU/g albumin); (c) Glycated albumin levels (mg/mL); (d) Fructosamine levels (mol/g protein); (e) Fluo-AGE levels (AU/g); (f) AGE levels (μg/mL). The horizontal bars in the box represent the median; the box boundaries represent the 25th and 75th percentile values. $^{***}P < 0.001$ and $^{**}P < 0.01$ indicate a significant difference versus ND group.

Table 2: Correlation coefficients between glycation, oxidation, and glycoxidation biomarkers for the total panel.

	Thiols (μmol/g)				
Thiols (μmol/g)	—				
		IMA/ALB (g/L)			
IMA/ALB (g/L)	0.186	—			
			Fluo-AGE (AU)		
Fluo-AGE (AU)	0.015	0.219*	—		
				AGE (ng/mL)	
AGE (ng/mL)	0.017	0.048	0.028	—	
					GA (mg/L)
GA (mg/L)	0.183	0.302***	0.036	0.399***	—
Fructosamines (mol/g)	0.130	0.430***	0.032	0.135	0.492***

Glycation parameters: fructosamine, glycated albumin (GA); oxidation parameters: ischemia-modified albumin (IMA), thiols; glycoxidation parameters: advanced glycation end-products (AGE), fluorescent advanced glycation end-products (Fluo-AGE). Total panel includes diabetic and nondiabetic patients ($N = 106$). Univariate correlation coefficients and significance between different biochemical parameter values compared by peer were calculated according to Pearson's method: $^{*}p < 0.05$, $^{***}p < 0.001$.

Table 3: Clinical and biochemical characteristics of the diabetic patients.

	NVD	VD	
N	$n = 31$	$n = 44$	
Male Sex (%)	$n = 7$ (22.6)	$n = 15$ (34.1)	$p = 0.270$
	Average +/− SD	*Average +/− SD*	*p value*
Age	56.2 +/− 2.3	60.8 +/− 1.4	0.070
BMI	31.0 +/−1.3	29.7 +/− 0.8	0.036
Total protein (g/L)	71.1 +/− 4.8	70.3 +/− 4.4	0.369
Albuminemia (g/L)	41.9 +/− 2.8	42.5 +/− 2.8	0.156
Fructosamine (mol/g)	4.68 +/− 0.78	4.84 +/− 0.77	0.392
HbA1c (%)	8.9 +/− 0.2	9.1 +/− 0.2	0.31
Glycemia (mmol/L)	9.0 +/− 2.9	9.2 +/− 3.1	0.754
Total cholesterol (mmol/L)	4.17 +/− 0.84	4.11 +/− 1.17	0.807
HDL-cholesterol (mmol/L)	1.13 +/− 0.30	1.12 +/− 0.28	0.882
LDL-cholesterol (mmol/L)	2.49 +/− 0.79	2.52 +/− 0.97	0.887
Triglycerides (mmol) (25–75th percentile)	1.34 (0.99–1.77)	1.49 (0.99–1.91)	0.520
ApoA (mmol/L)	1.30 +/− 0.22	1.30 +/− 0.22	1.000
ApoB (mmol/L)	0.89 +/− 0.21	0.88 +/− 0.28	0.866
Urine protein (g/L) (25–75th percentile)	0.09 (0.06–0.13)	0.10 (0.07–0.16)	0.370
Microalbuminuria (mg/L)	63.1 +/− 153.2	83.5 +/− 192.5	0.606

The panel of diabetic patients is divided into two groups: diabetic patients with and without vascular disease (resp., VD, $n = 44$, and NVD, $n = 31$).

and glycoxidation, only Fluo-AGE levels were found to be discriminatory between groups. Indeed, the Fluo-AGE levels were significantly higher in VD group than in NVD group ($p < 0.05$). However, the increases of IMA index ($p = 0.809$) and fructosamine levels ($p = 0.392$) in VD group were not statistically significant as shown in Figure 2.

The correlation coefficients analysis featured in Table 4 did not show any significant relationship between main biological parameters of oxidation and glycation. However and noteworthy enough, a significant correlation was established between glycated albumin and nonfluorescent AGE levels ($r = 0.4312$, $p < 0.0001$).

Because confounding factors such as sex, age, and BMI could influence both AGEs products and vascular complication occurrence, we performed a multivariate analysis (Table 5). Significant association between fluorescent AGE levels and personal history of microangiopathy was evidenced using univariate analyse. When adjusted for age, sex, and BMI, the association between fluorescent AGE levels and personal history of microangiopathy failed to reach significance ($p = 0.07$).

4. Discussion

Our study was designed to investigate a potential relationship between specific biomarkers of oxidation, glycation, and glycoxidation in diabetic patients and whether they could be associated with vascular complications related to diabetic

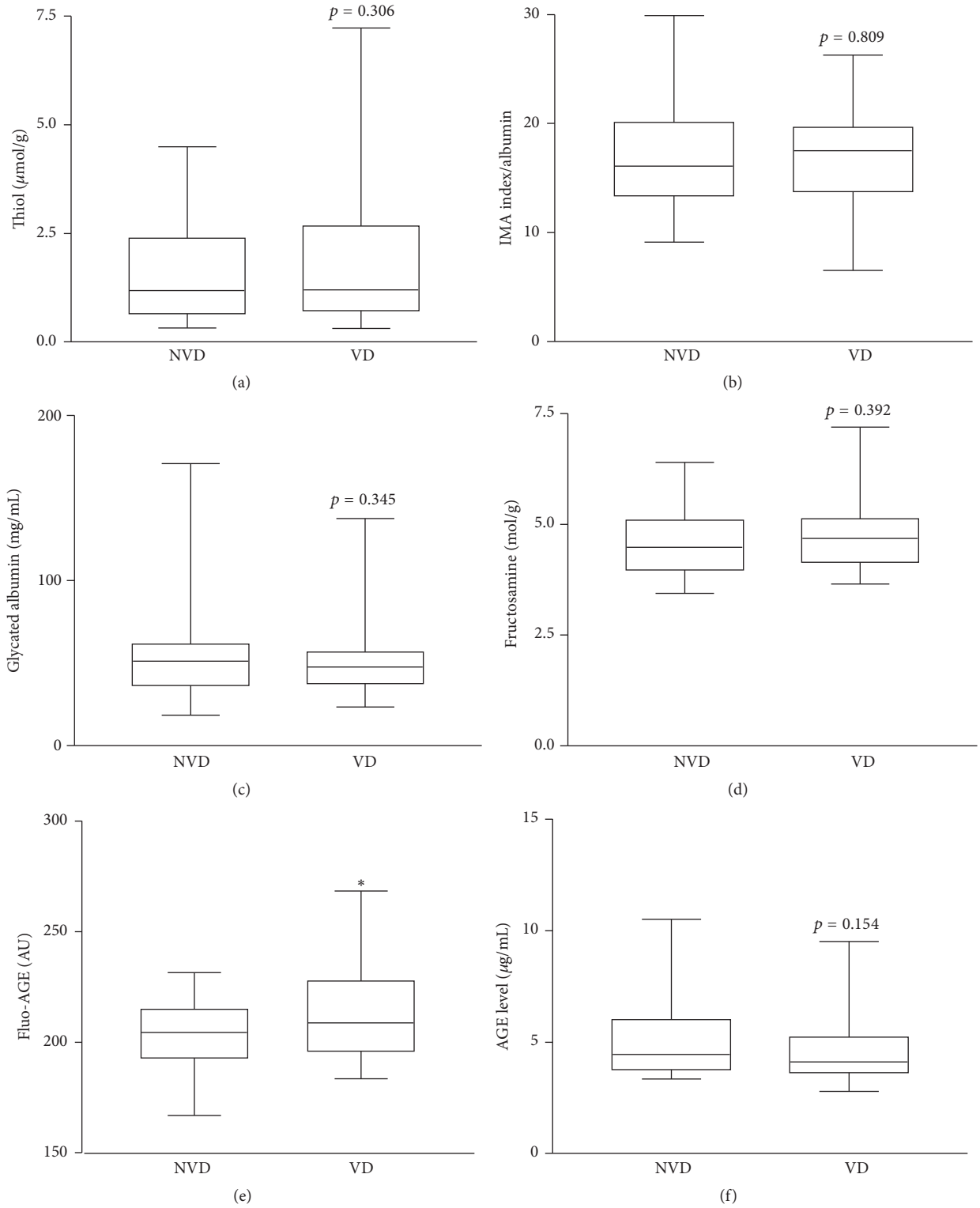

Figure 2: Box-plot distribution of biomarker values in the diabetic group with and without vascular disease groups (resp., VD and NVD). (a) Thiol levels (μmol/g protein); (b) IMA index (AU/g albumin); (c) Glycated albumin levels (mg/mL); (d) Fructosamine levels (mol/g protein); (e) Fluo-AGE levels (AU/g); (f) AGE levels (μg/mL). The horizontal bars in the box represent the median; the box boundaries represent the 25th and 75th percentile values. $^*P < 0.05$ indicates a significant difference versus NVD group.

Table 4: Correlation coefficients between glycation, oxidation, and glycoxidation biomarkers in the diabetic group.

	Thiols (μmol/g)				
Thiols (μmol/g)	—				
		IMA/ALB (g/L)			
IMA/ALB (g/L)	0.074	—			
			Fluo-AGE (AU)		
Fluo-AGE (AU)	0.068	0.011	—		
				AGE (ng/mL)	
AGE (ng/mL)	0.072	0.027	0.036	—	
					GA (mg/L)
GA (mg/L)	0.061	0.174	0.103	0.4312***	—
Fructosamines (mol/g)	0.151	0.181	0.012	0.110	0.230

Glycation parameters: fructosamine, glycated albumin (GA); oxidation parameters: ischemia-modified albumin (IMA), thiols; glycoxidation parameters: advanced glycation end-products (AGE), fluorescent advanced glycation end-products (Fluo-AGE). The panel includes diabetic patients with and without vascular disease ($N = 75$). Univariate correlation coefficients and significance between different biochemical parameter values compared by peer were calculated according to Pearson's method: $^{***}p < 0.001$.

Table 5: Associations between fluorescent AGE levels and personal history of vascular disease in univariate and multivariate analyses (*adjusted for age, sex, and BMI).

	Univariate odds ratio for vascular disease (IC 95%)	p value	Multivariate* odds ratio for vascular disease (IC 95%)	p value
Fluo AGE (per 1000 U)	1.32 (1.01–1.75)	0.04	1.35 (0.97–1.88)	0.07

microangiopathy and macroangiopathy. If some of these markers associated with diabetes were clearly correlated with one another (such as IMA with GA and fructosamine or GA with fructosamine), others (i.e thiols and AGEs) were independent. Biomarker of oxidation, early glycation, or advanced glycoxidation appeared independent when considering the sole diabetic group. In addition, these biochemical parameters provide nonredundant information to fructosamine and HbA1c assays.

T2DM is characterized by chronic hyperglycemia which can induce different molecular mechanisms involved in diabetes-associated metabolic disorders [26]. Through several pathways, hyperglycemia induces an increase of oxidative stress leading to an overproduction ROS and free radical species associated with an impairment of antioxidant defence systems [27]. The nonenzymatic glycation process occurring at higher rate in diabetic versus healthy subjects gives rise to the formation of early glycation products such as fructosamine which can evolve to AGEs [6]. In oxidative stress situations, these AGEs may result from oxidation reactions of early stage glycation products [28]. The overproduction of ROS contributes to structural, biochemical, and functional alteration of albumin resulting in a decrease of thiol levels associated with an increase of IMA index [9, 13]. In parallel, the nonenzymatic glycation process combined with oxidation reaction promotes the formation of early glycation products (fructosamine, Amadori products, or GA) and eventually of terminal products (AGEs) derived from albumin. The results presented here, indicating significant increases of IMA, GA, and fructosamine levels and decrease of plasmatic thiol level with type 2 diabetic patients, are in full accordance with the pathophysiological process occurring in a hyperglycemic context.

The absence of correlation between thiol and IMA levels is somehow surprising. Indeed, IMA index is related to the reduced binding capacity of HSA for Co^{2+} metal ions. It was already demonstrated that the structural integrity of HSA was essential for the binding between albumin and cobalt [13]. The alteration of plasma redox state in diabetic situation, characterized by a significant decrease in thiol levels, may have a direct impact on IMA levels. Compared to another study, the correlation between IMA and glycated albumin obtained here was also unexpected [29]. Indeed, this clinical study demonstrated that IMA was rather correlated with fasting glucose levels rather than with glycated albumin. In addition, IMA was also reported as an early marker of myocardial ischemia in patients after a percutaneous coronary intervention because IMA level variations were detected only few minutes after intervention and remain stable after few hours [30].

Among T2DM patients, glycated albumin was positively associated with AGE levels, reflecting that AGE formation is an early pathophysiological process involving glycated albumin. In addition, most of biomarkers displayed similar levels in patients with and without vascular disease. With a $p < 0.05$, the variation in fluorescent AGE level appeared to be detectable between both diabetic groups. This result appears to be in accordance with previous clinical study and fluorescent AGE could represent a relevant biomarker for the detection of vascular events in diabetic patients after validation in a broader clinical study. Indeed, a large scale clinical study has recently shown that the activation of the

receptor for advanced glycation end-products (RAGE) by AGE is implicated in the development of diabetes complications [31].

AGEs are heterogeneous and complex structures, which may represent important mediators of diabetic vascular complications. AGEs notably induce alterations of both functional and mechanical properties of tissue via crosslinking intracellular and extracellular proteins [32–34]. In support, some studies have shown correlations between serum AGE levels and the development/severity of vascular disease [35]. Besides, others failed to established associations of some specific plasma AGEs with CVD in individuals with normal glucose metabolism or T2DM [36, 37].

Because of their heterogeneous structures, the contribution of each AGE molecule to the pathophysiology remains unknown [38]. Among AGEs, fluorescent ones are crosslinking structures such as Pentosidine and Crossline and known to contribute to the development of vascular complications [39]. In particular, several studies in diabetic or hemodialysis patients reported a correlation between serum Pentosidine levels and arterial wall stiffness, a recognized component in the determination of vascular risk [40, 41]. Another study, evaluating the prognostic value fluorescent AGEs in the context of acute coronary syndrome, reported the association of fluorescent AGE levels and the occurrence of cardiac events during the follow-up period [42].

Although an increased value of IMA was found in diabetic group when compared with nondiabetics, this oxidative stress marker displayed no significant variations among the diabetic group. Contradictory results were reported in other studies of T2DM patients with peripheral arterial disease or patients who had stroke [43, 44]. In these studies, IMA and other glycation markers such as HbA1c were assessed in diabetic patients with recent or progressive vascular events while in the present study all patients with progressive or recent serious vascular complications such as myocardial infarction or cerebral vascular accident were excluded. This could partly account for the lack of difference and this should be considered as a limitation in the study. The absence of significant variations of IMA index in our study could also be due to the limited sensitivity of this parameter at early stage of vascular events. Our study is original since all patients presented a serious long-standing T2DM (duration of diabetes greater than 10 years and HbA1c > 7.5%) always associated with overweight or obesity (BMI > 25). The use of IMA index as an alternative indicator for determining vascular risk and oxidative stress has generated numerous studies [43, 45, 46].

A number of factors may account for the lack of significant differences between the diabetics with and without vascular complications in our study. First of all, very few cases of macroangiopathy were observed in the VD group that consisted essentially in patients with diabetic microangiopathy. Secondly, the sample size of our study probably limited the statistical power. Biomarkers may accumulate in the plasma due to renal insufficiency. The absence of data concerning glomerular filtration rate or creatinemia of our patients represents a weakness of this present work. At last, a limitation of this work is the cross sectional design which did not allow investigation of causal mechanisms connecting glycoxidation and vascular complications.

In order to evaluate the different biomarkers of oxidative stress and glycation as potential predictors of vascular disease in T2DM patients, a broad cohort study would be needed including the collection of biological samples and the vascular disease assessment. The carotid intima media thickness (IMT) determined by echo tracking [44] or the arterial stiffness estimation by pulse wave velocity (PWV) [47] could be considered as substitute criteria of vascular disease burden reflecting atherosclerosis.

Further studies remain highly warranted in order to reach a better understanding of the potential interest of glycated albumin, oxidative stress, and glycoxidation parameters in the monitoring of type 2 diabetic patients. Nonetheless, the present study brings new insights on fluorescent AGEs as a putative indicator for vascular event prediction in diabetic patients.

Abbreviations

ACB: Albumin-cobalt binding
AGE: Advanced glycation end-products
ACS: Acute coronary syndrome
BMI: Body mass index
CVD: Cardiovascular disease
CVA: Cerebrovascular accidents
Fluo-AGE: Fluorescent advanced glycation end-products
GA: Glycated albumin
IMA: Ischemia-modified albumin
MI: Myocardial infarction
nf-AGE: Nonfluorescent AGE
ROS: Reactive oxygen species
T2DM: Type 2 diabetes mellitus.

Conflicts of Interest

The authors hereby confirm that no conflicts of interest and no conflicts of financial interest exist.

Authors' Contributions

Conception and design of the study, acquisition of data, drafting the article, and final approval were done by Alexis Guerin-Dubourg; conception and design of the study, acquisition of data, drafting the article, and final approval were done by Maxime Cournot; acquisition of data, drafting the article, and final approval were done by Cynthia Planesse; conception and design of the study, acquisition of data, drafting the article, and final approval were done by Xavier Debussche; conception and design of the study, drafting the article, and final approval were done by Olivier Meilhac; conception and design of the study, drafting the article, and final approval were done by Philippe Rondeau; conception and design of the study, drafting the article, and final approval were done by Emmanuel Bourdon. Philippe Rondeau and Emmanuel Bourdon contributed equally to this work.

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Research Article

Red Cell Distribution Width Is Associated with All-Cause and Cardiovascular Mortality in Patients with Diabetes

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Background and Methods. Red cell distribution width (RDW) has emerged as a prognostic marker in patients with cardiovascular diseases. We investigated mortality in patients with diabetes included in the National Health and Nutrition Examination Survey, in relation to baseline RDW. RDW was divided into 4 quartiles (Q1: ≤12.4%, Q2: 12.5%–12.9%, Q3: 13.0%–13.7%, and Q4: >13.7%). *Results*. A total of 3,061 patients were included: mean age 61 ± 14 years, 50% male, 39% White. Mean RDW was 13.2% ± 1.4%. Compared with first quartile (Q1) of RDW, patients in Q4 were more likely to be older, female, and African-American, have had history of stroke, myocardial infarction, and heart failure, and have chronic kidney disease. After a median follow-up of 6 years, 628 patient died (29% of cardiovascular disease). Compared with Q1, patients in Q4 were at increased risk for all-cause mortality (HR 3.44 [2.74–4.32], $P < .001$) and cardiovascular mortality (HR 3.34 [2.16–5.17], $P < .001$). After adjusting for 17 covariates, RDW in Q4 remained significantly associated with all-cause mortality (HR 2.39 [1.30–4.38], $P = 0.005$) and cardiovascular mortality (HR 1.99 [1.17–3.37], $P = 0.011$). *Conclusion*. RDW is a powerful and an independent marker for prediction of all-cause mortality and cardiovascular mortality in patients with diabetes.

1. Introduction

Diabetes is associated with increased risk of microvascular and macrovascular complications [1–3]. Cardiovascular disease (CVD) is the leading cause of death among patients with diabetes accounting for 30–40% of deaths [4–6]. The risk of CVD can be modified using pharmacologic and nonpharmacologic measures [7–11]. Thus, it is important to accurately estimate the risk of cardiovascular disease to allocate resources and focus preventive measures among these high risk patients. While many risk scores have been devised to estimate the risk of cardiovascular disease among patients with diabetes, they often are difficult to incorporate in the clinical routine and have only modest discriminatory power [12, 13].

Red cell distribution width (RDW), a measure of variability in red blood cell size, is routinely measured in complete blood counts and is traditionally used to identify etiology of anemia. It is automatically calculated as standard deviation of mean corpuscular volume divided by mean corpuscular volume × 100%. Over the past decade, RDW has emerged as a prognostic marker in patients with CVD. Several studies have reported the prognostic power of RDW in patients with heart failure (HF) [14, 15] and coronary artery disease (CAD) [16–18], where it appears to be a powerful and independent marker of outcomes. Additionally, RDW has also been shown

to predict incident diabetes [19, 20], incident CVD, and mortality in community-dwelling subjects [21, 22].

Patients with diabetes have higher RDW than patients without diabetes [23, 24]. One prior study showed that, among patients with diabetes, RDW is associated with the presence of microvascular and macrovascular complications [25]. Whether RDW predicts mortality in patients with diabetes is not known. We sought to investigate the association between RDW with all-cause and cardiovascular mortality in a large representative cohort of noninstitutionalized patients with diabetes.

2. Methods

2.1. Dataset. NHANES is a program of studies designed to understand the health and nutritional status of adults and children in the US. This study was designed as a cross-sectional, repeated, multistage survey of noninstitutionalized US adults and children. This survey included questionnaires, physical examination, and laboratory testing. We included all adults (≥18 years) with self-reported diabetes mellitus, who were enrolled in the NHANES between 1999 and 2010, and have linkage to mortality data as described later (follow-up until 2011). All protocols were approved by the institutional review board at the National Center for Health Statistics (NCHS), and all participants provided informed consent.

2.2. Predictor Variable. Red cell distribution was measured from blood obtained from participants at the time of examination. RDW was measured using the Beckman Coulter MAXM instrument in the Mobile Examination Center. RDW was treated as continuous and categorical (quartile) variable in this analysis.

2.3. Outcomes. Mortality was identified through probabilistic linkage with the national death index using patient identifiers (e.g., social security number and date of birth) through 2011. The linkage is performed by the National Center for Health Statistics [26]. For this study, we identified all-cause mortality and cardiovascular mortality as defined by the 10th revision of International Classification of Diseases codes (I00 to I99).

2.4. Statistical Analyses. Continuous variables are presented as means (standard deviations) or median (25th–75th percentiles) as appropriate. Categorical variables are presented as numbers and percentages. No assumptions were made for missing variables. Logistic regression models were used to identify the association between RDW and the underlying comorbidities (self-reported myocardial infarction (MI), self-reported stroke, and chronic kidney disease (CKD): defined as estimated glomerular filtration rate (eGFR) of less than 60 ml/mins per 1.73 m^2 using the CKD-EPI equation [27]), with adjustment for (defined a priori) age, gender, race, hemoglobin, SBP, smoking, cholesterol, and insulin use. Unadjusted survival analyses were performed with Kaplan-Meier method and compared using Log Rank (Mantel-Cox) test. The follow-up duration was estimated using the reverse Kaplan-Meier method described by Schemper and Smith [28]. Cox proportional hazard models were adjusted for the following covariables (defined a priori): model 1: age, gender, race, and hemoglobin; model 2: model 1 + HF, MI, stroke, malignancy, CKD, BMI, SBP, and cholesterol; model 3: model 2 + oral antidiabetics, insulin, statins, ACE/ARBs, and diuretics. Hazard ratios and 95% confidence intervals for the adjusted and unadjusted models were estimated using Cox proportional hazard models. Penalized smoothing splines were also performed using the spline and survival packages in R to visualize the association of continuous RDW with hazards of mortality. All tests were two-sided and $P < 0.05$ was considered statistically significant. All analyses were performed on Statistical Package for Social Sciences (SPSS, version 21) and R-Package 3.3.1 for Windows.

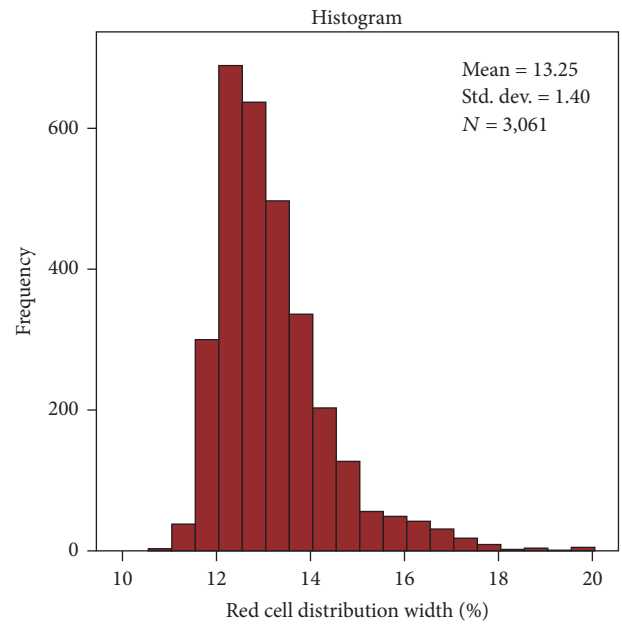


Figure 1: Distribution of red cell distribution width in the study cohort.

3. Results

A total of 3061 patients were included: mean age 61 ± 14 years, 50% male, 39% White. Mean RDW was 13.2% ± 1.4%. Distribution of RDW is shown in Figure 1. Compared with first quartile (Q1) of RDW, patients in Q4 were more likely to be older (Q1 versus Q4, age 58 versus 65 years, $P < 0.001$), female (48% versus 57%, $P < 0.001$), and African Americans (13% versus 42%, $P < 0.001$), have had history of stroke (6% versus 15%, $P < 0.001$), MI (6% versus 20%, $P < 0.001$), and HF (3.8% versus 21%, $P < 0.001$), and have CKD (13% versus 35%, $P < 0.001$), albuminuria (median ACR 0.12 versus 0.25, $P < 0.001$), and higher c-reactive protein (0.24 versus 0.45 mg/dL, $P < 0.001$), but there was no difference in the prevalence of retinopathy ($P = 0.77$) (Table 1).

RDW correlated negatively with hemoglobin ($r = -0.47$, $P < 0.001$), mean red cell volume ($r = -0.29$, $P < 0.001$), and mean red cell hemoglobin ($r = -0.37$, $P < 0.001$), eGFR ($r = -0.25$, $P < 0.001$), and positively with c-reactive protein

Table 1: Baseline characteristics of patients with diabetes by quartiles of RDW (NHANES 1999–2010).

Characteristics	Red cell distribution width (%)				P value*
	Q1 (n = 839) ≤12.4%	Q2 (n = 736) 12.5%–12.9%	Q3 (n = 737) 13.0%–13.7%	Q4 (n = 749) >13.7%	
Age (years), mean ± SD	58 ± 14	62 ± 14	64 ± 12	65 ± 13	<**0.001**
Women, n (%)	401 (48%)	347 (47%)	351 (48%)	424 (57%)	<**0.001**
African-American, n (%)	109 (13%)	141 (19%)	213 (29%)	314 (42%)	<**0.001**
Ever smoker (%)	412 (50%)	372 (51%)	372 (51%)	408 (55%)	0.22
History of MI, n (%)	50 (6%)	73 (10%)	105 (14%)	147 (20%)	<**0.001**
History of HF, n (%)	31 (4%)	50 (7%)	91 (13%)	157 (21%)	<**0.001**
History of malignancy, n (%)	104 (13%)	85 (12%)	96 (13%)	130 (17%)	**0.017**
History of stroke, n (%)	50 (6%)	62 (9%)	89 (12%)	112 (15%)	<**0.001**
BMI (kg/m^2), mean ± SD	30 ± 6	31 ± 7	33 ± 7	33 ± 9	<0.001
SBP (mmHg), mean ± SD	131 ± 21	134 ± 22	133 ± 21	135 ± 23	**0.001**
Hemoglobin (g/dL), mean ± SD	14.5 ± 1.4	14.1 ± 1.4	13.8 ± 1.4	12.8 ± 1.7	<**0.001**
eGFR (ml/min per 1.73 m^2), mean ± SD	90 ± 24	83 ± 25	80 ± 26	71 ± 31	<**0.001**
CKD (eGFR < 60), n (%)	105 (13%)	143 (19%)	173 (24%)	262 (35%)	<**0.001**
Retinopathy, n (%)	183 (22%)	168 (23%)	165 (22%)	188 (25%)	0.77
UACR, median [IQR]	0.12 [0.06–0.37]	0.14 [0.07–0.44]	0.17 [0.07–0.61]	0.25 [0.08–0.96]	<**0.001**
Total cholesterol (mg/dL), mean ± SD	197 ± 45	192 ± 45	188 ± 44	188 ± 54	<**0.001**
Hemoglobin A1c (%), median [IQR]	7.1 [6.2–8.6]	7.0 [6.1–8.3]	6.9 [6.2–8.0]	6.8 [6.1–7.7]	<0.001
Random blood glucose (mg/dL), median [IQR]	139 [106–205]	136 [106–186]	128 [101–172]	125 [98–170]	<0.001
CRP (mg/dL), median [IQR]	0.24 [0.10–0.54]	0.25 [0.12–0.55]	0.34 [0.14–0.74]	0.45 [0.20–1.03]	<**0.001**
Medications					
Oral antidiabetic, n (%)	532 (63%)	515 (70%)	530 (72%)	520 (69%)	0.002
Metformin, n (%)	334 (40%)	354 (48%)	357 (48%)	309 (41%)	<0.001
Insulin, n (%)	185 (22%)	167 (23%)	208 (28%)	234 (31%)	<**0.001**
Aspirin, n (%)	35 (4%)	29 (4%)	39 (5%)	40 (5%)	0.43
ACE/ARB, n (%)	390 (47%)	376 (51%)	430 (58%)	412 (55%)	<**0.001**
Statins, n (%)	283 (34%)	307 (42%)	337 (46%)	326 (44%)	<**0.001**
Diuretic, n (%)	181 (22%)	191 (26%)	260 (35%)	321 (43%)	<0.001
Number of deaths					
All-cause mortality	110 (13.1%)	134 (18.2%)	146 (19.8%)	238 (31.8%)	—
Cardiovascular mortality	30 (3.6%)	39 (5.3%)	48 (6.5%)	63 (8.4%)	—

*MI: myocardial infarction, HF: heart failure, SBP: systolic blood pressure, eGFR: estimated glomerular filtration rate, CKD: chronic kidney disease, UACR: urinary albumin to creatinine ratio, CRP: C-reactive protein, ACE: angiotensin convertase enzyme inhibitor, and ARB: angiotensin receptor blockers.

(r = 0.20, P < 0.001) and urine albumin : creatinine ratio (r = 0.15, P < 0.001).

RDW was associated with underlying diabetes-related complications (MI, stroke, and CKD). Compared with Q1 and after adjusting for age, gender, race, hemoglobin, SBP, smoking, cholesterol, and insulin use, patients in Q4 had higher risk of MI (OR 3.17 [2.17–4.64], P < 0.001), stroke (OR 1.12 [1.03–1.22], P = 0.006), and CKD (OR 1.13 [1.05–1.21], P = 0.002). Diabetes-related complications increased with RDW: MI (OR 1.22 [1.13–1.33] per 1% increment in RDW, P < 0.001), stroke (OR 1.12 [1.03–1.22] per 1% increment in RDW, P = 0.006), and CKD (OR 1.13 [1.05–1.21] per 1% increment in RDW, P = 0.002). Table 2 shows the odds ratio of diabetes-related complications in unadjusted and adjusted models.

After a median follow-up of 6 years, 628 patients died (29% of CVD). Compared with Q1, patients in Q4 were at increased risk for all-cause mortality (HR 3.44 [2.74–4.32], P < 0.001) and cardiovascular mortality (HR 3.34 [2.16–5.17], P < 0.001). Figure 2 depicts the Kaplan-Meier figures of all-cause and cardiovascular mortality by RDW quartile. After adjusting for 17 covariates, RDW in Q4 remained significantly associated with all-cause mortality (HR 2.39 [1.30–4.38], P = 0.005) and cardiovascular mortality (HR 1.99 [1.17–3.37], P = 0.011). Table 3 shows the multivariable adjusted models by

Table 2: Unadjusted and adjusted odds of underlying diabetes-related complications by RDW quartile.

	MI	Stroke	CKD	Retinopathy
	Odds ratio (95% confidence interval), *P* value			
Unadjusted				
Q2 versus Q1	1.72 [1.19–2.51], **P = 0.004**	1.44 [0.98–2.13], *P* = 0.063	1.69 [1.28–2.22], **P < 0.001**	1.06 [0.84–1.34], *P* = 0.64
Q3 versus Q1	2.59 [1.82–3.68], **P < 0.001**	2.16 [1.50–3.10], **P < 0.001**	2.14 [1.64–2.80], **P < 0.001**	1.03 [0.81–1.31], *P* = 0.80
Q4 versus Q1	3.82 [2.73–5.36], **P < 0.001**	2.76 [1.94–3.91], **P < 0.001**	3.76 [2.92–4.85], **P < 0.001**	1.20 [0.95–1.51], *P* = 0.14
Adjusted‖				
Q2 versus Q1	1.55 [1.05–2.30], **P = 0.027**	1.22 [0.81–1.82], *P* = 0.35	1.20 [0.88–1.65], *P* = 0.25	0.99 [0.77–1.28], *P* = 0.96
Q3 versus Q1	2.15 [1.48–3.12], **P < 0.001**	1.73 [1.18–2.55], **P = 0.005**	1.24 [0.91–1.70], *P* = 0.17	0.90 [0.69–1.18], *P* = 0.45
Q4 versus Q1	3.17 [2.17–4.64], **P < 0.001**	1.93 [1.30–2.86], **P = 0.001**	1.64 [1.20–1.11], **P < 0.001**	0.84 [0.64–1.12], *P* = 0.23

‖Adjusted for age, gender, race, hemoglobin, SBP, smoking, cholesterol, and insulin use. MI: myocardial infarction and CKD: chronic kidney disease.

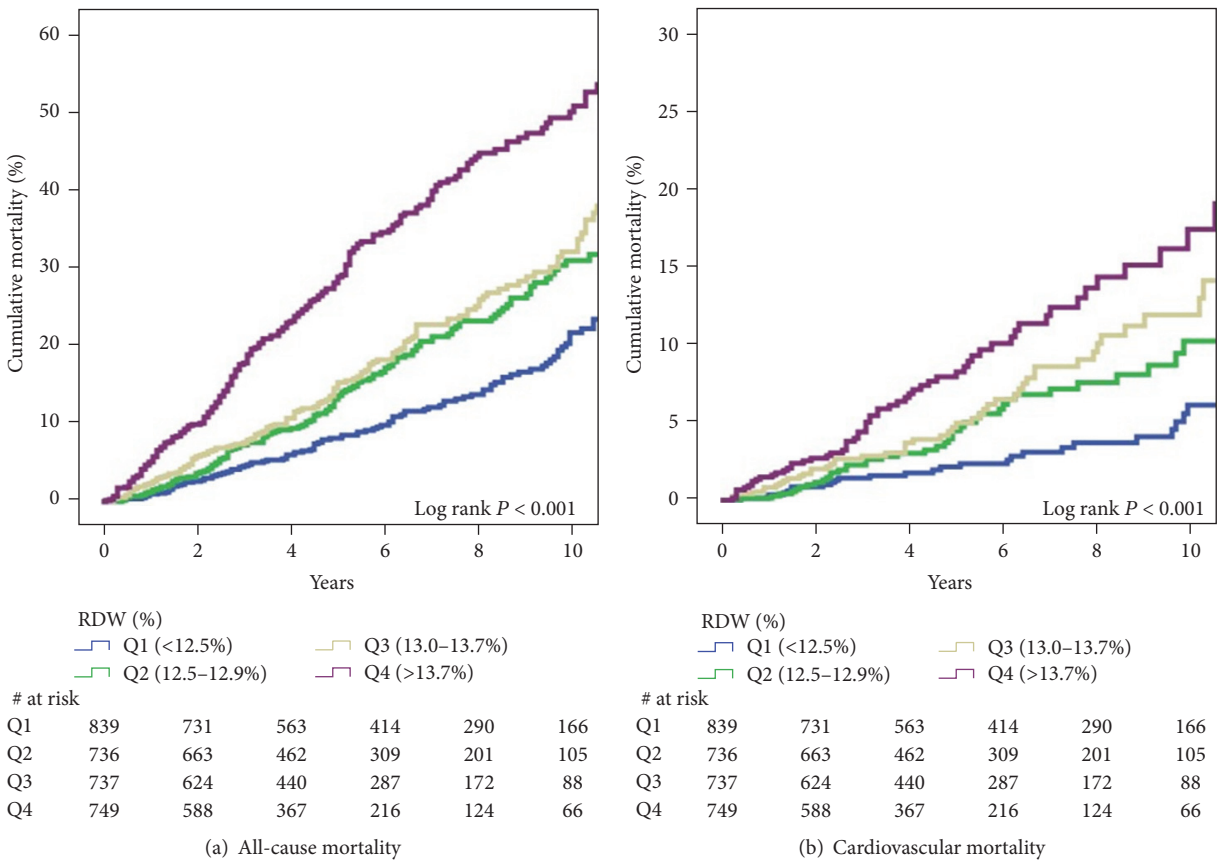


Figure 2: Kaplan-Meier curves of all-cause (a) and cardiovascular (b) mortality by quartiles of RDW. RDW: red cell width distribution.

quartile of RDW for all-cause and cardiovascular mortality. In a penalized smoothing spline, and compared with RDW of 11%, hazard ratio of all-cause mortality and cardiovascular mortality increased significantly until about RDW of 15%, with no further increase with higher values, Figure 3.

4. Discussion

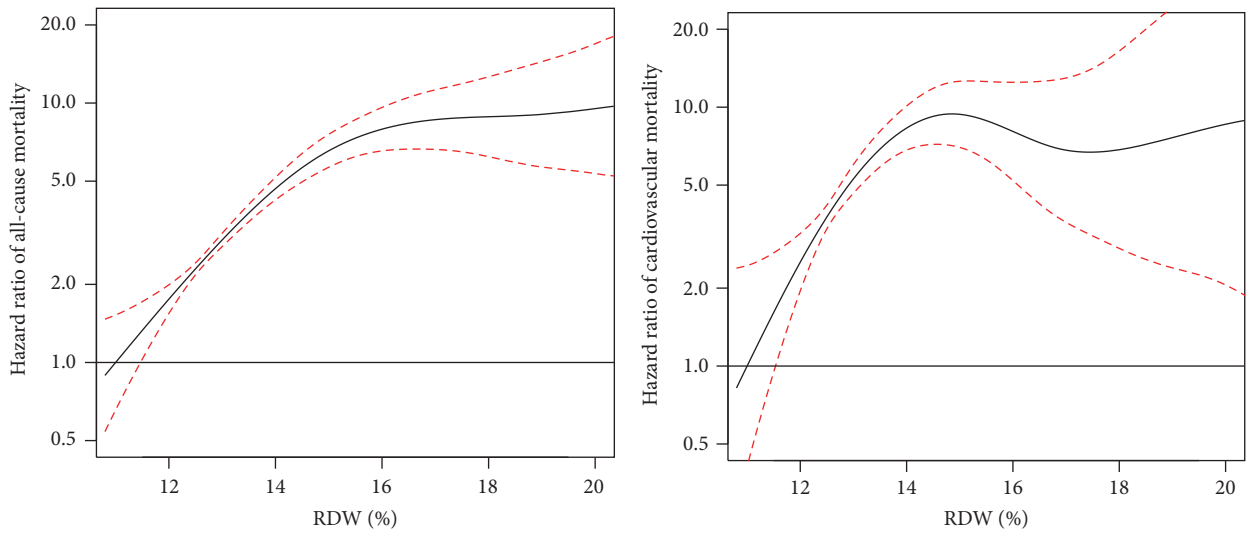
To our knowledge, this is the first study to evaluate the prognostic implications of RDW in community-dwelling patients with diabetes. We show that RDW is associated with underlying diabetes-related complications, namely, MI, stroke, and CKD. We also show that RDW is an independent and strong marker of cardiovascular and all-cause mortality in these patients.

Elevated RDW indicated high variability of erythrocyte size, which is a marker of ineffective erythropoiesis. Prior studies have identified an association between RDW and markers of inflammation such as Interleukin 6 [15], soluble tumor necrosis factor [29], iron mobilization (soluble transferrin receptor [15]), and oxidative stress [30]. All these mechanisms have been implicated in erythropoiesis and anemia.

Table 3: Association between RDW and all-cause and cardiovascular mortality.

	All-cause mortality		CV mortality	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Unadjusted				
Q2 versus Q1	1.54 [1.19–1.98]	**0.001**	1.64 [1.02–2.65]	**0.041**
Q3 versus Q1	1.79 [1.40–2.29]	<**0.001**	2.16 [1.37–3.41]	**0.001**
Q4 versus Q1	3.44 [2.74–4.32]	<**0.001**	3.34 [2.16–5.17]	<**0.001**
Per 1%	1.20 [1.16–1.23]	<**0.001**	1.15 [1.08–1.23]	<**0.001**
Model 1[‖]				
Q2 versus Q1	1.20 [0.93–1.55]	0.16	1.25 [0.77–2.02]	0.37
Q3 versus Q1	1.35 [1.05–1.74]	0.02	1.57 [0.99–2.50]	0.058
Q4 versus Q1	2.37 [1.85–3.03]	<**0.001**	2.22 [1.39–3.55]	**0.001**
Per 1%	1.16 [1.11–1.20]	<**0.001**	1.10 [1.01–1.19]	**0.032**
Model 2[†]				
Q2 versus Q1	1.17 [0.89–1.54]	0.27	1.23 [0.74–2.06]	**0.43**
Q3 versus Q1	1.25 [0.94–1.65]	0.12	1.37 [0.81–2.32]	0.24
Q4 versus Q1	2.03 [1.54–2.68]	<**0.001**	1.96 [1.16–3.31]	**0.012**
Per 1%	1.14 [1.09–1.20]	<**0.001**	1.09 [0.98–1.20]	0.13
Model 3[‡]				
Q2 versus Q1	1.26 [0.68–2.35]	0.47	1.30 [0.78–2.19]	0.32
Q3 versus Q1	1.66 [0.91–3.04]	0.098	1.41 [0.83–2.38]	0.21
Q4 versus Q1	2.39 [1.30–4.38]	**0.005**	1.99 [1.17–3.37]	**0.011**
Per 1%	1.09 [0.99–1.22]	0.094	1.08 [0.97–1.20]	0.15

[‖]Model 1: age, gender, race, and hemoglobin. [†]Model 2: Model 1 + HF, MI, stroke, malignancy, CKD, BMI, SBP, and cholesterol. [‡]Model 3: Model 2 + oral antidiabetics, insulin, statins, ACE/ARBs, and diuretics.



(a) All-cause mortality (b) Cardiovascular mortality

Figure 3: Association between continuous RDW with all-cause (a) and cardiovascular (b) mortality. RDW: red cell width distribution.

While higher RDW is associated with lower hemoglobin, in this analysis the mean hemoglobin across the 4 quartiles did not fall into the "anemia" range.

Our study confirms a prior analysis of the association between RDW and underlying diabetes-related complications in cross-sectional study design. In a study of 2,497 patients with diabetes enrolled in the previous version of NHANES (NHANES III, 1988–1994), third and fourth quartiles of RDW were associated with increased odds of myocardial infarction (OR 2.45 [95% CI 1.13, 5.28]), stroke (OR 2.56 [1.21–5.42]), and nephropathy (OR 2.33 [1.42–3.82]), but not retinopathy [25]. Another smaller study showed that

RDW is independently associated with underlying microalbuminuria in patients newly diagnosed with diabetes [31]. Our study validates these observations in an independent cohort. Because of the cross-sectional design, however, the temporal relationship of these events cannot be ascertained.

The prognostic role of RDW in diabetes is incompletely understood. To our knowledge, only one study investigated the prognostic impact of RDW in patients with diabetes with CAD. Among 560 patients with diabetes and stable CAD who underwent percutaneous coronary intervention, high RDW (≥13.1%) was independently associated with all-cause mortality (HR 2.56 [1.12–6.62], $P = 0.025$) [32]. Our findings generalize the prognostic role of RDW in predicting not only all-cause mortality but also cardiovascular mortality in a larger cohort of patients with diabetes with low prevalence of cardiovascular disease. It is important to note that in our fully adjusted model (model 3, Table 3), only 4th quartile of RDW (>13.7%) was consistently associated with increased cardiovascular and all-cause mortality. This is likely related to a threshold effect within RDW that limits our conclusions in mid-range RDW (12.4%–13.7%), as these levels (quartiles 2 and 3) were only associated with cardiovascular and all-cause mortality in partially adjusted models.

We also show that the risk of cardiovascular and all-cause mortality increased with RDW at levels considered within the normal limit in many clinical laboratories. As shown in Figure 3, hazards of cardiovascular mortality and all-cause mortality start increasing at about RDW of 12%. It is thus important to reconsider the traditional cutoffs if this test is to be used for prognostic and cardiovascular risk predictions.

Measurement of RDW often incurs no additional cost as it is a part of the routine automated complete blood counts and can provide prognostic information beyond traditional factors. Future studies should investigate the incremental value of adding RDW to predictive risk scores for cardiovascular disease in patients with diabetes. RDW could be used to select a cohort of patients enriched for poor outcomes for prevention trials. As shown in Figure 3, RDW higher than 15% was associated with approximately 10-fold increase in mortality, thus serving as a powerful tool for risk stratification in this high risk group. The change in RDW could potentially serve as a surrogate marker for all-cause and cardiovascular mortality that could be used in pilot studies of primary and secondary prevention of cardiovascular disease in diabetes.

Our study has few limitations that need to be acknowledged. We lack vital data on the duration of diabetes, type of diabetes, and etiology, as well as the prevalence of other cardiovascular risk factors such as dyslipidemia or hypertension. Cause specific mortality is derived from death certificates and thus may not be accurate in classifying etiology, particularly in out-of-hospital deaths. The dataset also does not capture incident cardiovascular events, such as myocardial infarctions or strokes, that would be important to describe in relationship to RDW. Additionally, data on factors related to RDW such as nutritional deficiencies (e.g., iron, folate, or vitamin B12) or blood transfusions are not consistently available in the dataset.

5. Conclusion

Red cell distribution is a powerful and an independent prognostic marker for prediction of all-cause mortality and cardiovascular mortality in patients with diabetes. Further studies should focus on incorporating RDW in risk prediction models in diabetes.

Disclosure

Analyses, interpretations, or conclusions presented in this manuscript do not represent the views of the National Center for Health Statistics (NCHS), who is responsible only for the initial data. The contents are solely the responsibility of the authors and do not necessarily represent the views of the Qatar National Research Fund.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

The Landscape of Glucose-Lowering Therapy and Cardiovascular Outcomes: From Barren Land to Metropolis

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The choice of glucose-lowering therapy (GLT) has expanded to include 11 different classes in addition to insulin. Since the 2008 Food and Drug Administration guidance for industry and mandate of demonstrating cardiovascular (CV) safety prior to any new drug approval, there were several trials primarily conducted to establish that goal. Some had neutral effects, while there were positively beneficial outcomes with more recent studies. Hospitalization for congestive heart failure has also been a heterogeneous finding among the different classes of GLT, with drug outcomes ranging from risky to beneficial. The current review selectively focuses on the evidence for CV outcomes for each class of GLT and summarizes the existing guidelines with regard to these drugs in heart disease. Moreover, it illustrates the dynamic status in the development of evidence. Finally, the review enables healthcare providers to formulate a plan for hypoglycemic therapy which will optimize CV health, in a patient-centered manner.

1. Introduction

Case. A 58-year-old man presents to the endocrine clinic for Type 2 Diabetes (T2D) management. He was diagnosed with T2D 8 years priorly, after hospitalization for acute myocardial infarction (MI), for which he underwent urgent revascularization with a stent placed in the left anterior descending artery. Since then, he has been on medical therapy and lifestyle management. He stopped smoking, decreased his Body Mass Index (BMI) from 32 to 29.5 kg/m^2, and started exercise two to three times per week. His work is stressful as a regional manager and involves frequent travel. He has no other significant comorbidities. His T2D medication is metformin 2000 mg per day. In addition, he is on antiplatelet therapy, angiotensin receptor blockade, beta blockade, and high intensity statin.

His studies reveal hemoglobin A1c (HbA1c) 8.3%; ALT 45 IU/L; creatinine 0.9 mg/dL with eGFR > 60 mL/min/1.73 m^2, potassium 4.5 mmol/L, glucose 135 mg/dL, LDL-C 62 mg/dL, HDL-C 42 g/dL, triglyceride 180 mg/dL, total cholesterol 140 mg/dL, hemoglobin of 13 g/dL with MCV of 92 fl, and urine microalbumin/creatinine ratio of 40 mg/g. Echocardiogram done one year ago shows moderate to good ejection fraction of 55% with mild apical hypokinesia.

The management of this patient falls under the American Diabetes Association (ADA) guidelines for comprehensive diabetes care, in terms of lifestyle recommendations and preemptive measures such as immunization updates, dental checks, cancer screening, and complications prevention [1]. However, the specific management of his hyperglycemia raises the question on how to improve his metabolic control in a manner to optimize cardiovascular (CV) health.

Due to the rapid accumulation of knowledge in the field of glycemic control and CV outcomes, there have been a number of reviews shedding different perspectives on this topic, within the past two years [2–8]. The current paper comprehensively assesses the existing classes of glucose-lowering therapy (GLT) with analysis of the available CV

data, so that the clinician can make informed patient-centered recommendations, supported by an updated body of evidence.

2. Historical Overview of Diabetes Therapy and Cardiovascular Effects

The pharmacologic therapy for T2D began in 1921 with the landmark discovery of insulin, followed by the availability of sulfonylureas (SU) and biguanides in the 1950s [9]. Guidelines for T2D therapy were largely shaped by the United Kingdom Prospective Diabetes Study (UKPDS), which has demonstrated that targeting an HbA1c of 7.0% as compared to 7.9%, in subjects newly diagnosed with T2D, reduced microvascular complications by 25% [10]. Macrovascular endpoints were more difficult to establish; in the strict glycemic control group, only the subgroup of overweight subjects on metformin had a significantly lower rate of CV events and mortality [11]. Because of the rapid growth of the epidemic and the multiplicity of pathophysiologic mechanisms in T2D, the development of new classes of drugs continued in an accelerated manner [9].

Prior to 2008 and largely guided by the UKPDS findings during the preceding decade, the FDA requirements for approval of a new hypoglycemic agent relied on demonstration of efficacy in glucose-lowering using HbA1c as a surrogate marker of vascular endpoints, granted no major adverse effects of the drug were observed in preclinical and clinical studies [12, 13]. The trials were typically short in duration and tended to exclude subjects with preexisting CV disease or renal insufficiency [13]. However, the vast majority of morbidity and mortality in T2D is a result of CV complications. Furthermore, diabetes drugs are typically consumed for many years, and any untoward late CV effect of a drug would likely be missed in these phase 3 clinical trials. One such example was the suspicion that was raised in 2007 regarding rosiglitazone and increased CV events [14]. Rosiglitazone, approved in 1999, was the most widely prescribed hypoglycemic agent at the time of the controversy due to its promising insulin-sensitizing profile. More clinical evidence on rosiglitazone is described under Thiazolidinediones. One additional unexpected finding which occurred during the same time period as the rosiglitazone controversy was the outcome of three large trials which showed that intensive glycemic control either provided no macrovascular benefit [15] or was associated with increased mortality [16]. Therefore, the use of HbA1c was no longer a valid intermediate marker of macrovascular outcomes.

All of the above factors have led the FDA to mandate evidence of CV safety prior to approval of any new potential GLT. Specifically, the drug had to demonstrate noninferiority in CV outcomes, with an upper bound hazard ratio of 1.3 at 95% confidence interval (CI), in order to be approved. Alternatively, the drug could show a noninferiority HR = 1.8 with conditional approval pending demonstration of CV safety at 1.3 in postmarketing studies [17]. As a result, trials testing new hypoglycemic therapies after December 2008 have tended to be much more homogenous and comparable in nature: subjects included had more CV risk factors including renal insufficiency, and trial duration was longer; the drug was tested against placebo on a background of "standard of care" T2D therapy; finally in the phase 3 testing of most drugs, the trial had as primary outcome major CV endpoints, which were independently adjudicated.

3. Available Hypoglycemic Classes

Along with insulin, there are currently 11 different classes of FDA-approved GLTs. The body of evidence on CV safety for drugs which became available after 2008 tends to be more robust, as a result of the changed FDA requirements. Nonetheless, data does exist for most other classes and will be reviewed below.

3.1. Sulfonylureas (SUs). SUs are blockers of the ATP-sensitive potassium channel of the beta islet cell and as such promote secretion of insulin [8]. The main risk of SUs is hypoglycemia and weight gain, especially when aiming for tight glucose control. They may also be associated with more rapid beta cell failure [18].

The earliest prospective, randomized double-blinded study to report on CV effects in T2D therapy is the University Group Diabetes Program conducted on 823 subjects assigned to the first-generation SU tolbutamide, lifestyle, insulin, or phenformin [19]. There was increased mortality in the tolbutamide group, and even though the study was criticized for not being powered enough, first-generation SUs were replaced by newer agents which cause less hypoglycemia. The second large body of evidence came from the 10-year follow-up of the UKPDS, whereby subjects initially randomized to the intensive glucose arm and who received the SUs chlorpropamide, glibenclamide, or glipizide had reduction of 15% in MI and 13% in overall mortality compared to the conventional group [20]. The latter was achieved despite the HbA1c becoming similar in both groups after trial completion and averaging 7.8% during the 10-year follow-up. While the target HbA1c of less than 7% in UKPDS was beneficial, the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial cast doubt as to the possibility of achieving tighter glycemic control of HbA1c less than 6.5% safely [16]. In this study conducted on 10250 adults with T2D, the group on intensive glycemic control using predominantly insulin, thiazolidinedione, or glimepiride had higher mortality than the conventional group, despite achieving an HbA1c of 6.4% versus 7.5%, respectively [16]. It is important to note that the mortality could not be linked to any single drug class, despite the higher use of thiazolidinediones, secretagogues, and insulin, in the intensive arm. Rather, subjects in the ACCORD trial were on average 10 years older (mean age 62 years) with a mean duration of diabetes of 10 years compared to the UKPDS subjects who were newly diagnosed with T2D. Therefore, the conclusion from that trial was mainly that tighter control in advanced diabetes using classes which predispose to hypoglycemia and weight gain may be deleterious, on the macrovascular level. However, some concerns were appeased when a similar population as that of the ACCORD was tested in the pharmaceutically initiated Action in Diabetes and Vascular Disease: Preterax and Diamicron

MR Controlled Evaluation (ADVANCE) trial. In this study, 11140 adults with T2D (mean age of 66 years and mean duration of disease of 8 years) were randomly assigned to either intensive glucose control with gliclazide or standard care for 5 years. By trial completion, the group on gliclazide had HbA1c of 6.5% versus 7.3% in the standard group and also had a lower primary composite outcome, mainly driven by reduction in nephropathy [21]. The benefits were sustained in a 5-year follow-up after the trial finish [22]. Most recently, a meta-analysis which included 47 randomized trials using second- or third-generation SUs against placebo or as add-on to metformin revealed no increase in CV risk [23]. However, the trials were not primarily designed to assess CV outcomes, and the study did not extract information on duration of disease nor presence of underlying CV risk, which constitutes a major limitation to generalizing these results.

3.2. Biguanides. Available since 1972, but FDA approved since 1994, metformin is the only existing compound in the class and has stood the test of time with continued benefits described. Metformin's main glucose-lowering effect is through reduction of hepatic gluconeogenesis. In addition, more mechanisms have been described on other parts of the gastrointestinal tract such as increased intestinal glucose utilization, increased glucagonlike peptide-1 levels, altered bile acids, and altered microbiome [24].

The CV benefit of metformin was first demonstrated in UKPDS where the overweight group on intensive glucose control had 39% reduction in MI rate [10]. In addition, in the 10-year follow-up of UKPDS, the long-term benefit for all subjects in the intensive arm on metformin was shown by reducing MI by 33% and all-cause mortality by 27% as compared to conventional control [20].

3.3. Alpha-Glucosidase Inhibitors (AGis). As the name implies, compounds available in this class—acarbose (FDA 1995), miglitol (FDA 1996), and voglibose (developed in Japan and available since 1994)—inhibit the enzyme responsible for breakdown of oligosaccharides into disaccharides at the intestinal brush border [8]. They do not cause hypoglycemia and target postprandial glucose. The main side-effect is gastrointestinal intolerance.

Acarbose has one completed placebo-controlled, randomized trial which assesses progression to T2D and the development of major CV events. In the STOP Non-Insulin Dependent Diabetes Mellitus (STOP-NIDDM) trial, with 1429 adults with impaired glucose tolerance and high CV risk, acarbose given over a mean of 3.3 years significantly reduced acute MI rate as well as the composite of macrovascular events with a drop of 49% relative risk (HR = 0.51; 95% CI: 0.28–0.95) and an absolute risk reduction of 2.5% [25]. Of note is the 24% dropout rate twice as high in the acarbose arm because of gastrointestinal intolerance. Voglibose was evaluated in a placebo-controlled, randomized trial on 859 adults with impaired glucose tolerance and recent acute MI in Japan. The trial ABC (Alpha-glucosidase-inhibitor Blocks Cardiac Events in Patients with Myocardial Infarction and Impaired Glucose Tolerance) was terminated after a two-year period due to total lack of difference between the 2 groups, in terms of CV outcomes. Of note is that the dropout rate was only 3%, as opposed to the large rate seen with STOP-NIDDM trial [26].

Further evidence is expected from the ongoing Acarbose Cardiovascular Evaluation (ACE) trial. This study will assess CV outcomes in more than 6000 adults over the age of 50 years with CV disease and impaired glucose tolerance, on acarbose versus placebo for a mean of 4 years. The primary results are expected to be announced in the fall of 2017 [27].

3.4. Meglitinides. Meglitinides are secretagogues which bind the same ATP-dependent potassium channel as SUs, but with shorter onset and duration of action [8]. They are metabolized and, as such, cause less hypoglycemia than SUs, especially in renal disease.

There are two agents available for use, repaglinide and nateglinide, FDA approved since 1997 and 2001, respectively. Studies assessing meglitinides and CV effects are limited. Because these agents target postprandial hyperglycemia, their cardioprotective effect may be similar to other compounds which reduce postmeal glucose excursions such as acarbose in the STOP-NIDDM trial. Nateglinide was used in the Long-Term Study of Nateglinide plus Valsartan to Prevent or Delay Type II Diabetes Mellitus and Cardiovascular Complications (NAVIGATOR) a placebo-controlled trial of 9306 subjects at high CV risk and with impaired glucose tolerance. After a median of 5 years, there was no reduction in the incidence of T2D nor in the composite outcome of CV disease. There were, however, more hypoglycaemia cases in the nateglinide arm [28].

3.5. Thiazolidinediones. Thiazolidinediones (TZDs) are insulin sensitizers which have ubiquitous effects on the liver, skeletal muscle, and adipose tissue through peroxisome proliferator-activated receptor gamma binding [29]. Known side-effects are fluid retention, weight gain, anemia, fractures, and exacerbation of heart failure [8]. Two compounds—rosiglitazone (FDA 1999) and pioglitazone (FDA 2001)—are available. A warning regarding pioglitazone and bladder cancer was issued in 2010, and the drug should not be prescribed in case of unexplained hematuria or active bladder cancer [30]. Both agents are contraindicated in subjects at risk of heart failure.

CV concern was raised with rosiglitazone 8 years after marketing, when a meta-analysis of 42 trials showed a 43% increase in the risk of MI (86 versus 72 events) and a 64% increase in death from CV disease (39 versus 22 deaths), even though the latter did not reach significance [14]. The study was criticized for not reporting absolute risk which was only 0.2% higher with rosiglitazone and, more importantly, for having excluded trials which showed no events, that is, 4 studies from the infarction analysis and 19 from the mortality analysis [31]. Nonetheless, rosiglitazone came under scrutiny and its use was heavily restricted by the FDA until the results of the trial designed primarily to assess CV risk came out. The Rosiglitazone Evaluated for Cardiovascular Outcomes in oRal agent combination therapy for type 2 Diabetes (RECORD) trial is an open-label study of 4447 subjects randomized to rosiglitazone versus a comparator

group of either metformin or SU, for a mean of 5.5 years. The inclusion criteria did not include high CV risk, and the event rate was relatively lower in both groups for MI (68 versus 60), for all-cause mortality (88 versus 96), and for stroke (50 versus 63), in rosiglitazone versus metformin/SU, respectively. There was no difference in the composite or individual endpoints [32]. There were higher rates of fatal and nonfatal heart failure (61 versus 29 subjects). The results of this pharmaceutical-initiated trial were further reaffirmed by independent review of adjudication [33] and the prescribing restriction was lifted in 2013. However, the trial was limited by a relatively high dropout rate of 18% and by the lack of blinding [34]. Rosiglitazone use remains limited among physicians.

Pioglitazone, the other compound available in this class, was also scrutinized when concerns about rosiglitazone were raised. However, a trial with primary CV endpoints was reassuring: the PROspective PioglitAzone Clinical Trial in macroVascular Events (PROactive) enrolled 5238 patients with T2D and established macrovascular disease, randomized to pioglitazone versus placebo, in addition to standard of care. Strangely, the trial was terminated early, after a 3-year follow-up, even though there was no difference in the primary 7-point composite outcome. However, a secondary outcome of nonfatal MI, stroke, or death was lower in the pioglitazone arm with 301 out of 2605 versus 358 out of 2633, for pioglitazone and placebo, respectively [35]. The authors were criticized for reporting a nonpredefined secondary outcome when the primary was negative [36]. Nonetheless, the reduction was consistent across all 3 components of the 3-point major adverse CV events (MACE). In line with these findings was a meta-analysis of 19 trials on pioglitazone which showed a risk reduction in MI, stroke, or death by 18% (HR = 0.82; 95% confidence interval [CI], 0.72–0.94; $p = 0.005$). The authors note that data from PROactive trial constitutes the bulk of events; nonetheless, the results from other trials in the meta-analysis were consistent [37]. As expected, there was a higher rate of heart failure (16% versus 11.5%); however, it did not result in increased mortality.

3.6. Amylin Analogues or Amylinomimetics. Pramlintide (FDA 2005) is an incretin cosecreted with insulin, which suppresses glucagon and delays gastric emptying [8]. As such, it targets postprandial glucose and does not induce hypoglycemia if used as monotherapy. However, its use is recommended as add-on to insulin, in which case cautious glucose monitoring is necessary to prevent severe hypoglycemia. Its mechanism of action (targeting postprandial glucose) would suggest favorable CV outcome; however no studies exist. Its use has been limited by twice-daily injections, relatively high cost, and guidelines which narrow its use to a mere add-on to prandial insulin [8].

3.7. Bile Acid Sequestrants. Colesevelam is the only hypoglycemic agent approved for such use in this category because it incidentally lowers HbA1c by 0.5% [8]. It obtained FDA approval, in January 2008, for use in T2D as an adjunct to diet and exercise. Its mechanism of action is unclear but may decrease intestinal glucose absorption. Its main side-effects are gastrointestinal.

The only study reporting on CV outcomes was a retrospective chart review in subjects with T2D and dyslipidemia, comparing those on colesevelam ($n = 847$) to those on ezetimibe ($n = 3384$). After adjustment for any baseline differences, fewer subjects on colesevelam had the primary CV event (HR = 0.58, $p = 0.004$). However, the authors themselves concluded that change in clinical practice cannot be made based on this study alone due to the limitation of a retrospective design [38].

3.8. Dopamine Agonists. An immediate-release formulation of bromocriptine is postulated to restore the circadian peak of dopaminergic activity in the hypothalamus and as such to decrease hepatic gluconeogenesis and insulin resistance [39]. Bromocriptine-QR (FDA 2009) is administered within two hours of waking up. Its main side-effects are nausea, dizziness, and orthostasis.

In a primary CV endpoint placebo-controlled trial, bromocriptine or placebo was administered over 12 months to 3070 subjects with T2D, in addition to standard therapy. A quarter of patients had preexisting CV disease. Bromocriptine reduced the composite outcome which included MI, stroke, revascularization, hospitalization for cardiac cause, and death to 32 versus 37 events (HR = 0.60; 95% CI: 0.37–0.96). However, the study was limited by a large number of subjects stopping the drug prior to final visit: 47% in bromocriptine group and 32% in the placebo group [39].

3.9. Dipeptidyl Peptidase-4 Inhibitors (DPP-4is). DPP-4 inhibitors increase endogenous levels of glucagonlike peptide-1 (GLP-1) and as such act as mild hypoglycemic oral agents. There are currently 4 FDA-approved agents: sitagliptin (FDA approved in 2006), saxagliptin (FDA approved in 2009), linagliptin (FDA approved in 2011), and alogliptin (FDA approved in 2013). Vildagliptin was mandated by the FDA in 2007 to conduct more trials in patients with renal insufficiency; there has been no reapplication for FDA approval since then, but it remains widely used in other parts of the world. Additionally, there are 2 once-weekly DPP-4i—trelagliptin and omarigliptin—both available in Japan. Side-effects from DPP-4 inhibitors have been described in postmarketing studies. The FDA issued a warning about a rare, but real, risk of pancreatitis for all agents in this class. The risk of pancreatic cancer remains a subject of debate [8]. Because the enzyme dipeptidyl peptidase exists in several forms and because DPP-4 activity is specifically exhibited by the cell surface protein CD26 of the T-lymphocyte, this class of drugs has also been associated with various disorders resulting from modulation of immune function such as autoimmune-related skin conditions (notably bullous pemphigoid), arthralgia, myalgia, and nasopharyngitis [40]. There are also agent-specific concerns on heart failure as described below.

CV safety was established for most currently available DPP-4 inhibitors. The first trial with CV endpoints, the Saxagliptin Assessment of Vascular Outcomes in Patients

with Diabetes Mellitus- Thrombolysis in Myocardial Infarction 53 (SAVOR-TIMI 53), enrolled 16 492 adults above 40 years of age, who had established CV disease or were at high risk for CV disease, who received saxagliptin or placebo, along with usual care, and who were followed for 2.1 years. At the end of trial, despite a small difference in the HbA1c of 0.2% in the intervention group (7.7 versus 7.9%), there was no difference in the primary endpoint of nonfatal MI, ischemic stroke, or CV death (613 in saxagliptin versus 609 in placebo group, HR = 1.00; 95% CI: 0.89–1.12, $p < 0.001$ noninferiority) [41]. However, there were more subjects who were hospitalized for nonfatal heart failure (289 in saxagliptin versus 228 in placebo group, HR = 1.27; 95% CI: 1.07–1.51, $p = 0.007$). Risk factors for heart failure were prior heart failure, a lower eGFR, and higher baseline pro-BNP levels [42]. Furthermore, the effect of the drug on heart failure was no longer seen one year into the trial.

In the second trial, the Examination of Cardiovascular Outcomes with Alogliptin versus Standard of Care (EXAMINE), 5380 men and women with acute coronary syndrome within the last 15–90 days were randomized to alogliptin or placebo, in addition to standard of care. After a median of 18 months, the difference in HbA1c between groups was only 0.3%, and there was no difference in the primary 3-point MACE (316 events for alogliptin versus 305 for placebo, HR = 0.96; upper boundary CI < 1.16). Hospitalization for heart failure occurred in 85 of alogliptin-treated patients versus 79 in the placebo group; however this number did not reach statistical significance [43].

In the third trial, Trial Evaluating Cardiovascular Outcomes with Sitagliptin (TECOS), 14,671 adults above 50 years of age, with established or at high CV risk, were assigned sitagliptin versus placebo in addition to standard of care [44]. After a median follow-up of 3 years, the HbA1c was 0.29% lower in the sitagliptin group; however, there was no difference in the primary 3-point MACE (839 events for sitagliptin versus 851 for placebo, HR = 0.98; 95% CI: 0.88–1.09, $p < 0.001$ for noninferiority). There were 228 subjects hospitalized for heart failure versus 229, in sitagliptin and placebo groups, respectively. The latter clearly indicates there was no increased risk of heart failure exacerbation, in the case of sitagliptin.

As mentioned, there is no primary CV outcome trial for vildagliptin. However, a meta-analysis which included 69 studies on 28,006 subjects on vildagliptin versus a comparator found no increased risk of CV events or heart failure [45]. In a 12-month Vildagliptin in Ventricular Dysfunction Diabetes (VIVIDD) trial 254 patients with NYHA Classes I–III heart failure were randomized to vildagliptin or placebo. There were 13 admissions for heart failure in the vildagliptin group versus 10 in the placebo group. Although the number did not reach statistical significance, the end-diastolic volume was higher in those on vildagliptin, again reinforcing previous suspicions about the group [46].

A systematic review and meta-analysis on DPP-4 inhibitors found a suggestion of increased heart failure risk, primarily driven by SAVOR, EXAMINE, and VIVIDD trials [47]. One proposed physiologic explanation for the heart failure finding is the inhibitory effect of this class on glucagon, a positive inotropic hormone [48]. One other advanced theory is the inhibition of breakdown of Neuropeptide Y, also a substrate of DPP-4, leading to vasoconstriction [49]. However, given the lack of consistency of the study results, more data will be helpful to further elucidate the question of DPP-4 inhibition and effect on heart failure. The CARdiovascular Outcome study of LINAgliptin versus glimepiride in patients with Type 2 Diabetes (CAROLINA) has randomized 6051 subjects above 40 years of age with either high risk or preexisting CV disease to linagliptin or glimepiride; its results are anticipated in the middle of 2019 [50]. Out of the once-weekly DPP4is, trelagliptin does not have a CV trial linked to it. Omarigliptin was undergoing CV assessment in a trial expected in 2021; however, the trial was terminated by the company MSD earlier than schedule (in 2016), with the announcement that the decision was made for marketing purposes and not for medical reasons [51, 52].

In summary, the use of DPP-4 inhibitors in subjects with CV disease seems neutral in terms of event outcomes. There appears, however, to be a small signal for heart failure, especially in those at risk.

3.10. Glucagonlike Peptide-1 Receptor Agonists (GLP-1 RA). GLP-1 normally secreted by the ileum stimulates insulin release in a glucose-dependent manner, inhibits glucagon release, and suppresses appetite both centrally and by delayed gastric emptying [8]. The class has been available since 2005 with several compounds: exenatide (FDA approved in 2005 for the twice-daily injection, FDA approved in 2012 for the once-weekly one), liraglutide (FDA approved in 2014), dulaglutide (FDA approved in 2014), albiglutide (FDA approved in 2014), lixisenatide (FDA approved in 2016), and semaglutide (application to FDA submitted December 2016). Concerns as a class have mainly been pancreatitis and a common side-effect is nausea. More recently, there are reports about gallbladder disease with increased risk of cholecystitis [53]. They are used with caution in people at risk of medullary thyroid cancer. There are GLP-1 receptors on the heart and questions were raised in view of the DPP-4 inhibitor results on heart failure; there is a mild, but consistent, increase in heart rate with all GLP-1 agonists [48]. This effect may be heterogeneous among the different compounds, with the shortest-acting agent increasing the rate by 1–3 beats per minute, all the way to the once-weekly agents increasing it by 6–10 beats per minute [54]. Despite the concern about heart rate increase and about glucagon inhibition (similar to DPP-4 inhibitors), GLP-1 may also act as a potent inotropic agent off-setting its potential cardiac drawbacks [55].

The first study in class to examine CV risk was the Evaluation of LIXisenatide in Acute coronary syndrome (ELIXA) which randomized 6068 men and women with T2D, who had acute coronary syndrome within the preceding 180 days, to lixisenatide or placebo on a background of usual care. After a median of 25 months, there were 406 events in the lixisenatide group versus 399 in placebo (HR = 1.02, 95% CI: 0.89–1.17, $p < 0.001$ for noninferiority) [56]. There was a similar incidence of hospitalization for heart failure among both groups.

The second outcomes trial, Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER), randomized 9340 adults above 50 years of age, with established disease or at high CV risk, to liraglutide or placebo against standard of care for a mean of 3.8 years. The average HbA1c at baseline was 8.7% and duration of T2D 12.8 years. By the end of trial, there was only a mild drop in HbA1c in both groups and subjects on liraglutide had a 0.4% lower level [57]. However, there was a significant reduction in the 3-point MACE with 608 events versus 694 in liraglutide and placebo, respectively (HR = 0.87; 95% CI 0.78–0.97, $p = 0.01$ for superiority). The difference was mainly driven by death from CV causes. This started to become apparent after 18 months of exposure to the drug. There was no difference in hospitalization for heart failure. In subgroup analysis, the benefit was observed across all groups. Additionally, there was 22% less risk of nephropathy, which, by itself, represents reduced macrovascular hazard.

A third study in the Trial to Evaluate Cardiovascular and Other Long-Term Outcomes with Semaglutide in Subjects with Type 2 Diabetes (SUSTAIN-6) randomized 3297 subjects to the once-weekly semaglutide at 0.5 or 1.0 mg doses versus placebo. Again, more than 80% of subjects had established CV disease, and the others were at high risk with age above 50 years and duration of T2D of 13.9 years. Baseline HbA1c was 8.7% and the difference at the end of 2.1-year follow-up was 0.7 and 0.9%, for the 0.5 mg and 1.0 mg doses of semaglutide, respectively [58]. The primary outcome of 3-point MACE occurred in 108 on semaglutide and 146 subjects on placebo (HR = 0.74; 95% CI 0.58 to 0.95, $p = 0.02$ for superiority). The outcome started to diverge after an 18-month lag time, and it was mainly driven by nonfatal MI and nonfatal stroke. This remained consistent across subgroup analysis. Similar to the other two GLP-1 trials, there was no difference in heart failure, which occurred in only 3.6 and 3.3%, for semaglutide and placebo, respectively. Also, similar to the liraglutide trial, there was a 36% reduction in the risk of new or worsening nephropathy.

The same investigators, who conducted the latter 2 trials, propose that liraglutide and semaglutide may be effective in reversing or stabilizing atherosclerosis given the lag time to see effects and the consistency of the results across subgroups. In addition, for both liraglutide and semaglutide, there was a small increase in progression of retinopathy and in cholecystitis for liraglutide. No such side-effects were reported in the lixisenatide trial.

There are 3 other once-weekly GLP-1 agonists, with ongoing trials for CV safety. Dulaglutide in the Researching CV Events with a Weekly Incretin in Diabetes (REWIND) and exenatide once weekly in Exenatide Study of Cardiovascular Event Lowering (EXSCEL) trial are both expected at the end of 2018. Finally, albiglutide in the HARMONY OUTCOME trial is expected in 2019 [59].

3.11. SGLT2 Inhibitors. Sodium-glucose cotransport 2 (SGLT2) inhibitors partially block glucose reabsorption in the proximal renal tubule by binding to the SGLT2 transporter. Available SGLT2 inhibitors are highly selective for the SGLT2 receptor in the renal tubule. However, there may be minor effect on intestinal SGLT1 inhibition, affecting glucose absorption [8]. Efficacy on HbA1c lowering averages 0.6%. Other than glucose-lowering, SGLT2 inhibitors decrease systolic and diastolic blood pressure mildly. It is preferable not to initiate them if eGFR < 60 mL/min/1.73 mm^2 (<45 for empagliflozin), and it is recommended to discontinue them if eGFR falls persistently below 45 mL/min/1.73 mm^2 with their use. They increase the risk of urinary tract infection and genital candidiasis. There are postmarketing reports of euglycemic diabetic ketoacidosis associated with their use. One potential explanation is that SGLT2 transporters are present on the alpha islet cells of the pancreas, and their inhibition results in higher glucagon secretion [48]. In May 2017, the FDA issued a drug safety alert on canagliflozin being associated with twice as much risk of toe and foot amputations, as the placebo group [60]. The mechanism is still unclear; however subjects on canagliflozin tended to have more peripheral vascular disease.

The first agent to be approved by the FDA, canagliflozin, became available in 2013, based on pooled data from 9 studies on 10285 subjects which suggested no CV harm with HR = 0.91 (95% CI: 0.68–1.22) [61]. Primary CV endpoints have just been made available with the CANagliflozin cardioVascular Assessment Study (CANVAS). In this trial, the integrated renal and CV pool of 10142 participants were reported together to maximize the power. Adults, above 50 years of age with established CV disease or above 60 years with two or more risk factors, received canagliflozin 100 mg or 300 mg or placebo in a 1 : 1 : 1 ratio, and they were followed over a 3.6-year period. The average age was 63.3 years, average BMI was 32.0 Kg/m^2, and duration of T2D was 13.5 years. There was a significantly lower risk of the 3-point MACE in the canagliflozin group reported in absolute numbers as 26.9 versus 31.5 participants with an event per 1000 patient-years, conferring a HR = 0.86 (95% CI: 0.75 to 0.97; $p = 0.02$ for superiority); however, none of the three components were significant on their own. In contrast, hospitalization rate for heart failure was markedly lower in the canagliflozin group, with 5.5 versus 8.7 participants with an event per 1000 patient-years with HR of 0.67 (95% CI: 0.52–0.87) [62]. Furthermore, the benefit was observed within six months of entry into the trial and sustained throughout. Adverse events were lower overall in the canagliflozin group; however they were consistent with previous reports on SGLT2 inhibitors.

Dapagliflozin (FDA approved in 2014) was the first SGLT2 inhibitor to become available outside the USA. The initial submission to FDA in 2011 was refuted based on concern for bladder and breast cancer. However, after review of two additional years of data and an increase of 50% in patient-year exposure to dapagliflozin, an analysis of 11000 patients with T2D revealed reassuring CV safety profile [63]. A primary outcomes trial, the Dapagliflozin Effect on CardiovascuLAR Events-Thrombolysis in Myocardial Infarction 58 (DECLARE-TIMI 58), enrolled more than 17000 subjects in 2013, and results are anticipated for 2019 [64]. Until then, two published studies are in favor of dapagliflozin: a meta-analysis of the phase 2b/3 studies suggested no increase in mortality, nor in the 3-point MACE [65]. Even more favorably, a retrospective case-control open-cohort population-based study

reviewed all-cause mortality and CV events in 22,124 patients with T2D on dapagliflozin ($n = 4444$) or not on SGLT2i ($n = 17680$) and found a significant decrease in all-cause mortality of 8.4 versus 17.2 incidence rate per 1000 person-years with adjusted relative risk 0.50 (95% CI 0.33–0.75) in the dapagliflozin group versus the control, respectively [66]. Additionally, the difference in mortality persisted in subgroup analysis when examining the low risk population.

Empagliflozin (FDA 2014) also has a completed primary CV outcomes trial. The EMPA-REG study randomized 7020 adults with high CV risk or disease to empagliflozin 10 mg or 25 mg or placebo in addition to standard of care [67]. The population was very similar to the previously described primary CV outcome trials, with average age 63 years, average BMI of 30 Kg/m^2, and more than 50% subjects with duration of diabetes of more than 10 years (EMPA-REG). After a follow-up of 3.4 years, there was a significant decrease in the 3-point MACE occurring in 490 out of 4687 (10.5%) in the empagliflozin group versus 282 out of 2333 (12.1%) in placebo, HR of 0.86 (95% CI: 0.74–0.99; $p = 0.04$ for superiority). The effect was largely driven by death from CV cause. Hospitalization for heart failure occurred in 4.1% in the placebo group versus 2.7% in empagliflozin conferring 35% risk reduction. Both the CV mortality and heart failure benefits were observed as early as 6 months into the trial and were sustained [67]. Based on the trial results, the FDA has issued an additional approval for empagliflozin to reduce CV death in T2D in December 2016.

Possible explanations for the unanticipated early beneficial and powerful results were hemodynamic (increased natriuresis, decrease in blood pressure) and metabolic (decreased waist circumference and weight, HbA1c decrease of 0.4%) in nature [68]. However, similar changes seen with other agents did not yield the same benefits as observed with empagliflozin or canagliflozin. One suggested theory is that an increase in ketone levels may be beneficial to the myocardium, especially an ischemic myocardium, providing an alternative source of energy [69]. One additional SGLT2i molecule, ertugliflozin, is currently undergoing Cardiovascular Outcomes Following Ertugliflozin Treatment in Type 2 Diabetes Mellitus Participants with Vascular Disease (VERTIS CV) [70].

3.12. Insulin. The main side-effects of insulin—weight gain and hypoglycemia—have led to cautionary recommendations when used in patients with high risk of CV disease. Nonetheless, the beneficial effect of insulin on vascular prevention was demonstrated in the UKPDS whereby the group on intensive therapy had macrovascular benefit after 10 years of trial completion [20].

However, trials primarily designed to assess CV effect of insulin and aiming at similar glycemic control in both arms are scarce. The only existing placebo-controlled trial achieving this aim used insulin glargine in Outcome Reduction with Initial Glargine INtervention (ORIGIN) and randomized 12537 subjects (mean age 63.5 years) with existing or high risk CV disease and prediabetes (11.5%) or T2D to receive glargine versus standard of care. The mean duration of T2D and HbA1c were similar in both groups and were 5.5 years and 6.4%, respectively. By the end of the study, the HbA1c was 6.2% in the glargine group and 6.5% in the standard care, with more incidence of severe hypoglycemia occurring in the glargine group. After a 6.2-year follow-up, there were similar rates of 3-point MACE with 2.94 and 2.85 per 100 person-years, for glargine and standard care, respectively. There were 310 versus 343 hospitalizations for congestive heart failure in the glargine or standard care, respectively; however it did not reach significance with HR of 0.90 (95% CI: 0.77–1.05, $p = 0.16$) [71].

The DEVOTE trial (Comparing Cardiovascular Safety of Insulin Degludec versus Insulin Glargine in Patients with Type 2 Diabetes at High Risk of Cardiovascular Events) randomized 7637 with T2D at high CV risk to either glargine or degludec, for an average of 1.99 years. The mean age was 65.0 years, BMI was 33.0 Kg/m^2, HbA1c was 8.4%, and duration of disease was 16.4 years. The results demonstrated noninferiority of degludec as compared to glargine, with respect to 3-point MACE. There were lower rates of hypoglycemia, including severe hypoglycemia [72]. Given that hypoglycemia represents an undesirable effect, especially in CV disease, it is worth noting that a more concentrated formulation of glargine U-300 was compared to glargine U-100 in both T1D and T2D and revealed less nocturnal hypoglycemia for the same level of glycemic control [73].

The Hyperglycemia and Its Effect after Acute Myocardial Infarction on Cardiovascular Outcomes in Patients with Type 2 Diabetes Mellitus (HEART2D) study was designed to target postprandial glucose with short-acting insulin as compared to fasting glucose with basal insulin in patients with acute MI occurring within 3 weeks of randomization [74]. After a 2.7-year follow-up on 1115 subjects, both groups achieved target HbA1c of 7% and the group on short-acting insulin had lower postmeal glucose excursions. However, there was no difference in the incidence neither of primary CV events nor on congestive heart failure.

Finally, a systematic review and meta-analysis comparing the CV outcomes of insulin versus noninsulin therapy included 18 trials and 5546 composite events occurring similarly in both arms [75]. It is important to note that only two out of these 18 trials extended beyond two years.

In brief, glucose-lowering with insulin provides macrovascular benefits, and limited studies have demonstrated its safety with respect to noninsulin therapy. The type and duration of action of insulin does not seem to affect the CV outcome; however it does impact on rates of hypoglycemia. Therefore, regimens should be judiciously prescribed to minimize this risk in patients with CV disease.

Table 1 summarizes the primary outcome of the completed and reported trials of GLT and CV outcome, after the 2008 FDA mandate. Figure 1 illustrates the timeline of the trials conducted in T2D with the primary outcome of CV disease.

4. Non-GLT Therapy with Hypoglycemic Benefits

Sacubitril belongs to a new class of drugs used for heart failure, the neprilysin inhibitors. Neprilysin is an enzyme

Table 1: Summary of the completed trials of hypoglycemic therapy and cardiovascular outcome as a result of the 2008 FDA mandate.

Trial	Tested molecule	Comparator	Population	*N*	Duration (years)	Primary outcome	Hazard ratio
ORIGIN [71]	Glargine	Standard care	Prediabetes or T2D with CV risk	12537	6.2	3-point MACE	1.02 (0.94–1.11)
DEVOTE [72]	Degludec	Glargine	T2D and above 50 years with CV or renal, or above 60 years at risk	7637	2.0	3-point MACE	0.91 (0.78 to 1.06)
SAVOR [41]	Saxagliptin	Placebo	T2D and above 40 with CV disease or above 55 years at risk	16492	2.1	3-point MACE	1.00 (0.89–1.12)
EXAMINE [43]	Alogliptin	Placebo	T2D and acute coronary syndrome within 15-90 days	5380	1.5	3-point MACE	0.96 (≤1.16)
TECOS [44]	Sitagliptin	Placebo	T2D and above 50 years and established CV disease	14671	3.0	4-point MACE	0.98 (0.88–1.09)
ELIXA [56]	Lixisenatide	Placebo	T2D and patients with acute coronary syndrome within 180 days	6068	2.1	3- point MACE	1.02 (0.89–1.17)
EMPA-REG [67]	Empagliflozin	Placebo	T2D and age above 18 years with established CV disease	7020	3.1	3-point MACE	0.86 (0.74–0.99)
CANVAS [62]	Canagliflozin	Placebo	T2D and age above 30 years with CV or above 50 years with ≥ 2 risks	10142	3.6	3- point MACE	0.86 (0.75 to 0.97)
LEADER [57]	Liraglutide	Placebo	T2D and age above 50 years and CV disease or above 60 years at risk	9340	3.8	3-point MACE	0.87 (0.78–0.97)
SUSTAIN-6 [58]	Semaglutide	Placebo	T2D and above 50 years with CV disease or age above 60 years at risk	3297	2.1	3- point MACE	0.86 (0.74–0.99)
ACE [88]	Acarbose	Placebo	IGT and above 50 years with CV disease	6522	5.0	5-point MACE	0.98 (0.86–1.11)
EXSCEL [89]	Exenatide	Placebo	T2D adults with established CV disease 70% or at risk 30%	14752	3.2	3-point MACE	0.91 (0.83–1.00)

Notes. Overall, study population across studies represents a high cardiovascular risk, with average age ranging from 60 to 65 years (±SD 7–10 years), mean BMI range is from 28.7 to 32.8 kg/m^2, with diabetes duration ranging between 5.4 and 13.8 years, and average HbA1C range is from 6.4 to 8.7% (±0.8–1.5%). Table adapted from Table 1 from [2].

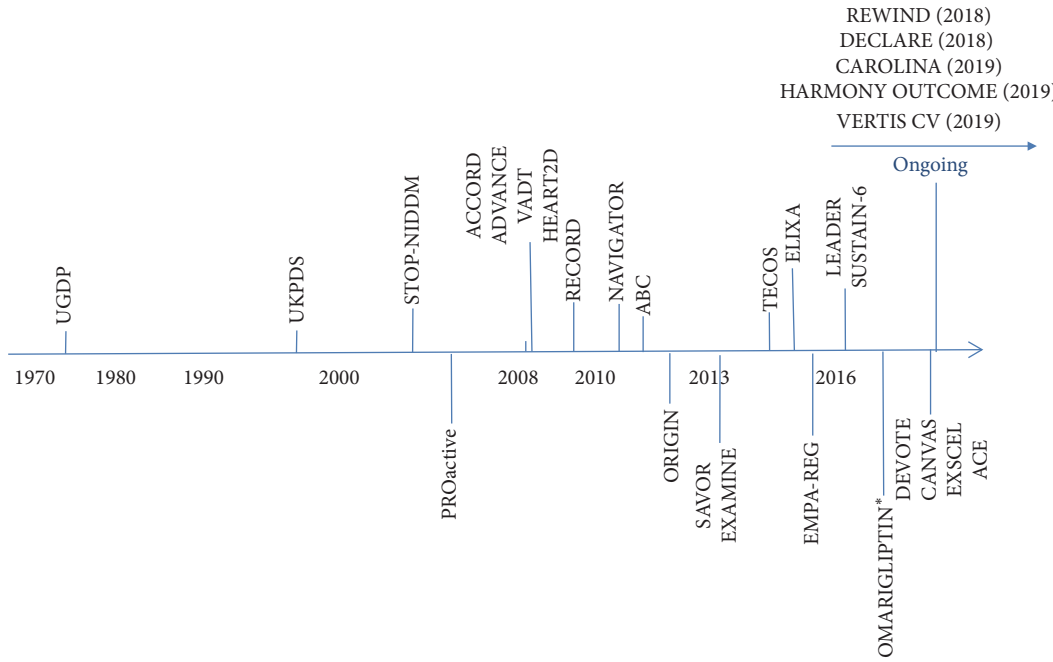

Figure 1: The landscape of cardiovascular trials in T2D. *Trial terminated.

expressed in the endothelium, cardiac myocytes, and adipocytes among other cells, responsible for the breakdown of a variety of vasoactive peptides such as natriuretic peptide, angiotensins I and II, bradykinin, and GLP-1. When combined with an Angiotensin Receptor Blocker (ARB), the sacubitril/valsartan net effect was shown to be favorable metabolically, with improved insulin sensitivity and glycemic control [76]. In the diabetes substudy of the PARADIGM-HF (Prospective Comparison of ARNI [Angiotensin Receptor-Neprilysin Inhibitor] with ACEI [Angiotensin-Converting-Enzyme Inhibitor]) to Determine Impact on Global Mortality and Morbidity in Heart Failure Trial, sacubitril/valsartan was compared with enalapril in 3778 subjects with New York Heart Association Classes II-IV heart failure and T2D. The combination in the original PARADIGM-HF study was overwhelmingly more powerful in reducing all-cause mortality, including death from CV cause, reducing hospitalization for heart failure, and improving physical symptomatology and functionality [77]. In addition, subjects with T2D had mildly lower HbA1c values (an absolute difference of −0.14%, 95% CI 0.06–0.23), needed to start insulin 29% less time than controls, and trended towards needing less oral hypoglycemic therapy [78].

The example of this new class of drugs, already FDA approved for the treatment of heart failure, blurs the margin between management of T2D and that of CV disease. These two conditions meet at many pathophysiological states and therefore ideally should be treated with drugs that have mutual beneficial effect.

5. Current Guidelines

The updated guidelines have taken into consideration recent evidence. Four points of agreement among the ones reviewed below are as follows:

(1) T2D management should be individualized with patient-specific glycemic targets.

(2) Lifestyle modification remains a mainstay therapy in the management.

(3) Metformin is the initial drug of choice.

(4) None of the guidelines reviewed incorporates the following 3 classes which are rarely used in T2D: bile acid sequestrants, amylin analogues, and dopamine agonists. Due to paucity of data, these will not be further discussed.

The Diabetes Australia 2016–2018 guidelines favor adding an SU, a DPP-4 inhibitor, or an SGLT2 inhibitor and if necessary, the addition of any of the above with GLP-1 agonists or insulin. The CV benefit observed with recent trials is mentioned, however not incorporated into a recommendation [79].

Diabetes Canada (previously called Canadian Diabetes Association) provided an interim update in November 2016 on glycemic management, whereby individuals are stratified according to the presence of CV disease or not. If present, then liraglutide or empagliflozin is to be considered next in line to metformin. If absent, then any of the classes of GLT would be suitable weighing in all the factors [80].

The National Institute for Health and Care Excellence (NICE) guidelines updated January 2017 mention as first intensification SUs, pioglitazone, or DPP-4 inhibitors, with certain restrictions on the use of pioglitazone [81]. The

addition of SGLT2 inhibitor is mentioned in instances when SUs are not tolerated or hypoglycemia is significant. The addition of GLP-1 agonist is to be considered as a third line whenever BMI is above 35 kg/m^2 or the use of insulin in those with a lower BMI would be restrictive occupationally. The CV outcomes observed with GLP-1 agonists and SGLT2 inhibitors are not incorporated into the algorithm.

The American Association of Clinical Endocrinologists (AACE) 2017 provides an algorithm with a hierarchical addition of GLT, with respect to the order of class suggested. After metformin, GLP-1 analogues and SGLT2 inhibitors would be second and third drugs, respectively. Whereas all classes are proposed as potential additions in case the SGLT2 inhibitors or GLP-1 analogues are not used, the guidelines point out that the use of TZDs, secretagogues, or insulin should proceed with caution. However, insulin is definitely recommended when triple therapy fails. Even though the guidelines emphasize patient individualization, they do no differentiate between CV risk or not when going through the hierarchy [82].

In contrast, the ADA in January 2017 incorporated new evidence as follows: after metformin all classes of drugs are possible second option if there is no increased CV risk. Even though they are provided as choices, meglitinides and AGis are not incorporated into the algorithm. Furthermore, if CV disease is established, then empagliflozin and liraglutide are to be considered due to the demonstration of benefit [1].

Finally, in terms of cardiology societies, the American Heart Association's last update on prevention of CV disease in adults with T2D was in 2015, jointly with the ADA. After metformin, the guidelines favor adding pioglitazone and acarbose. However, they were formulated prior to the most recent positive studies and therefore require an update before they can be followed [83]. The European Society of Cardiology (ESC May 2016) guidelines on chronic and acute heart failure do mention empagliflozin favorably after metformin and caution regarding insulin, TZDs, and secretagogues [84].

The new results of sacubitril/valsartan trial and GLP-1 RA and SGLT2i studies will likely shape the future management of T2D in CV disease, and upcoming society guidelines will likely be even more closely intertwined and multidisciplinary in nature.

6. Summary

From our own synthesis of the trial findings, we propose the following steps, as shown in Figure 2: firstly, lifestyle recommendation and the addition of metformin should remain the first step and the backbone of management of T2D; secondly, one needs to assess for cardiac risk; in case heart failure is present, then SGLT2i is added preferentially after metformin, followed by GLP-1 RA. We make a footnote regarding the glycemic benefit of neprilysin inhibitors/ARB combination, without recommending its addition for glycemic control primarily at this point. In case there is no heart failure risk but there is concern for atherosclerotic disease, then either SGLT2i or GLP-1 RA is recommended after metformin. Thirdly, if additional glucose-lowering is required, then gliclazide, pioglitazone, DPP-4 inhibitors, and basal insulin would be favored options. The upcoming ACE trial results, if positive, may also propel acarbose into an equally viable option. As a fourth step, if neither heart failure nor CV risk is present, then glycemic control can be achieved with any of the classes mentioned above, with special attention paid to patient risk factors and knowledge of side-effect profile such as risk of ketosis, pancreatitis, cholecystitis, osteoporosis, genital infections, and bladder cancer. Cost, feasibility, risk of hypoglycemia, and long-term glycemic control are factors to be considered (Figure 2). Cost consideration, in particular, would apply to the newer agents such as SGLT2i, DPP-4i, GLP-1 RA, and the "designer" insulin.

The Glycemia Reduction Approaches in Diabetes: A Comparative Effectiveness Study (GRADE) is an ongoing trial of 4–7 years aimed at comparing SU, insulin, DPP-4 inhibitor, and GLP-1 agonist for glycemic control and durability, which should help shed further light into the algorithm stratification [85]. In addition, whether the benefit of a drug is a class effect or a molecule effect remains to be proven within the next few years. To make the conclusion more complex, it is very difficult to make evidence-based recommendations regarding combinations. For example, despite the individual benefit of GLP-1 RA and SGLT2i, one cannot conclude that their combination will yield the same benefit. If glucagon is central to the advantage observed with SGLT2 inhibitors, then lowering it with GLP-1 may be detrimental. Only trials with the combination may be able to address this point. One such trial using once-weekly exenatide and dapagliflozin did show beneficial metabolic endpoints after 28 weeks of use [86]. However, the cost of such study of combination and CV assessment may be prohibitive. So, despite the accumulating evidence, the fine-tuning of glycemic control will always draw on the "art" of medicine, as well as its science. Last but not least, CV prevention in T2D is multifactorial and attention should be paid to lipids, smoking cessation, blood pressure control, obesity, and albuminuria. The benefit of multifactorial intervention was shown again by the extension of the STENO study, whereby 7.8 years of metabolic control increased lifespan by 7.9 years and delayed CV events by 8.1 years [87].

7. Concluding Remarks

The amount of knowledge over the past decade on glucose-lowering and CV effects has improved significantly. The choice of glucose-lowering medication has widened and remains patient-centered based on risk profile and potential benefit. The current review of the CV outcomes of all the available drugs with a perspective on the historical evolution of diabetes therapy keeps the available classes in context. The choice of drugs is likely to evolve further with refinement of our current knowledge. There were great strides made since the 10-year follow-up on the UKPDS showing CV risk reduction with improved glycemia. Therefore, addressing pharmacotherapy of T2D judiciously as highlighted in this paper should be carried out as part and parcel of overall patient well-being.

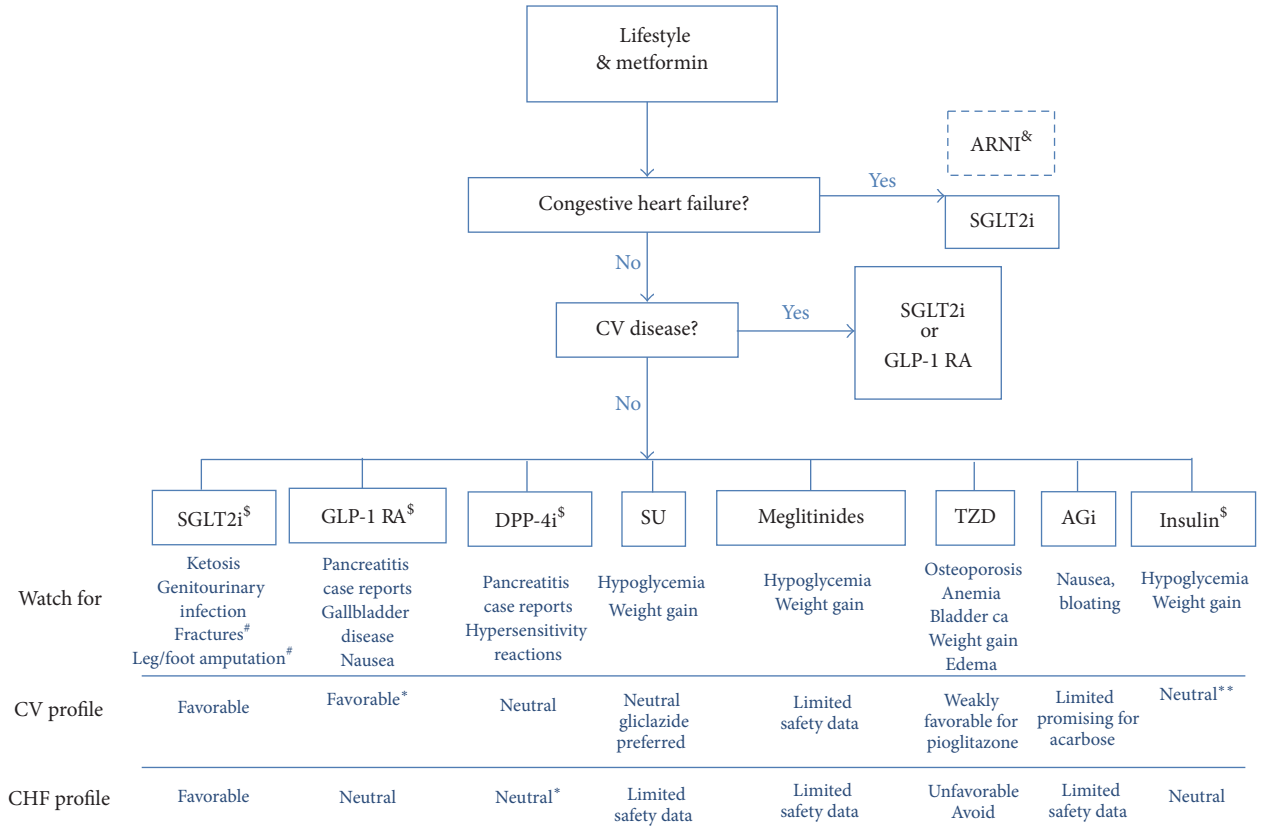

Figure 2: Suggested algorithm for pharmacotherapy in T2D. *Agent-specific. **Grade A evidence with glargine and degludec. #Effect reported for canagliflozin. &Not among the FDA-approved classes of GLT, however potential advantage in heart failure. $Cost may be a significant consideration. SGLT2i: sodium-glucose cotransport 2 inhibitor; GLP-1 RA: glucagonlike peptide-1 receptor agonist; DPP-4 i: dipeptidyl peptidase-4 inhibitor; SU: sulfonylurea; TZD: thiazolidinedione; AGi: alpha glucosidase inhibitor; ARNI: Angiotensin Receptor blocker Neprilysin Inhibitor.

Nomenclature

Abbreviations

AACE: American Association of Clinical Endocrinologists
ADA: American Diabetes Association
AGi: Alpha glucosidase inhibitor
AHA: American Heart Association
ARB: Angiotensin Receptor Blocker
ARNI: Angiotensin Receptor Blocker Neprilysin Inhibitor
BMI: Body Mass Index
CI: Confidence interval
CV: Cardiovascular
DPP-4i: Dipeptidyl peptidase-4 inhibitor
FDA: Food and Drug Administration
GLP-1 RA: Glucagonlike peptide-1 receptor agonist
GLT: Glucose-lowering therapy
HbA1c: Hemoglobin A1c
MACE: Major Adverse Cardiovascular Events
MI: Myocardial infarction
NICE: National Institute for Health and Care Excellence
SGLT2i: Sodium-glucose cotransport 2 inhibitor
SU: Sulfonylurea
T2D: Type 2 Diabetes
TZD: Thiazolidinedione.

Trial Acronyms

ABC: Alpha-glucosidase-inhibitor Blocks Cardiac Events in Patients with Myocardial Infarction and Impaired Glucose Tolerance
ACE: Acarbose Cardiovascular Evaluation
ACCORD: Action to Control Cardiovascular Risk in Diabetes
ADVANCE: Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation
CANVAS: CANagliflozin cardioVascular Assessment Study

CAROLINA: CARdiovascular Outcome study of LINAgliptin versus glimepiride in patients with Type 2 Diabetes
DECLARE-TIMI 58: Dapagliflozin Effect on CardiovascuLAR Events-Thrombolysis in Myocardial Infarction 58
DEVOTE: Comparing Cardiovascular Safety of Insulin Degludec versus Insulin Glargine in Patients with Type 2 Diabetes at High Risk of Cardiovascular Events
ELIXA: Evaluation of LIXisenatide in Acute coronary syndrome
EMPA-REG: Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes
EXAMINE: Examination of Cardiovascular Outcomes: Alogliptin versus Standard of Care in Patients with Type 2 Diabetes Mellitus and Acute Coronary Syndrome
EXSCEL: Exenatide Study of Cardiovascular Event Lowering
GRADE: Glycemia Reduction Approaches in Diabetes: A Comparative Effectiveness Study
HARMONY OUTCOME: Effect of Albiglutide, When Added to Standard Blood Glucose-Lowering Therapies, on Major Cardiovascular Events in Subjects with Type 2 Diabetes Mellitus
HEART2D: Hyperglycemia and Its Effect after Acute Myocardial Infarction on Cardiovascular Outcomes in Patients with Type 2 Diabetes Mellitus
LEADER: Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results
NAVIGATOR: Long-Term Study of Nateglinide + Valsartan to Prevent or Delay Type II Diabetes Mellitus and Cardiovascular Complications
ORIGIN: Outcome Reduction with Initial Glargine INtervention
PARADIGM-HF: Prospective Comparison of ARNI [Angiotensin Receptor-Neprilysin Inhibitor] with ACEI [Angiotensin-Converting-Enzyme Inhibitor]
PROactive: PROspective PioglitAzone Clinical Trial in macroVascular Events
RECORD: Rosiglitazone Evaluated for Cardiovascular Outcomes in oRal agent combination therapy for type 2 Diabetes
REWIND: Researching Cardiovascular Events with a Weekly Incretin in Diabetes
SAVOR-TIMI 53: Saxagliptin Assessment of Vascular Outcomes Recorded in patients with diabetes mellitus-Thrombolysis in Myocardial Infarction 53
STENO-2: Intensified multifactorial intervention and cardiovascular outcome in type 2 diabetes
STOP-NIDDM: Stop Non-Insulin Dependent Diabetes Mellitus
SUSTAIN-6: Trial to Evaluate Cardiovascular and Other Long-Term Outcomes with Semaglutide in Subjects with Type 2 Diabetes
TECOS: Trial Evaluating Cardiovascular Outcomes with Sitagliptin
UGDP: University Group Diabetes Program
UKPDS: United Kingdom Prospective Diabetes Study
VADT: Veterans Affairs Diabetes Trial
VERTIS CV: Cardiovascular Outcomes Following Ertugliflozin Treatment in Type 2 Diabetes Mellitus Participants with Vascular Disease
VIVIDD: Vildagliptin in Ventricular Dysfunction Diabetes.

Additional Points

Postscript. After acceptance of the paper, two trials which were listed as pending were actually presented and published September 13 and 14, 2017, for ACE and EXSCEL, respectively [88, 89]. The ACE trial conducted on 6522 Chinese adults above age of 50 years with known CV disease and impaired glucose tolerance was started in 2009 and completed in April 2017. The trial used an intermediate dose of acarbose of 50 mg three times daily, and it had a higher adherence rate of above 96% as compared to STOP-NIDDM. The results, however, were minimal for metabolic improvement in favor of acarbose, and the primary outcome (5-point MACE) showed no difference between the two groups (HR = 0.98; 95% CI: 0.86–1.11). The second aim of the study which was diabetes prevention did show an 18% reduction in the risk of T2D, a fact already known for this class [88]. The

EXSCEL trial was an event-driven trial which reached its target earlier than anticipated. This study was conducted on 14,752 adults with known CV disease (70%) or at high risk (30%) and T2D who were given once-weekly exenatide of 2 mg or placebo, with the standard of care, and they were followed for a median of 3.2 years. There was no difference in the 3-point MACE (HR = 0.91; 95% CI: 0.83–1.00, $p < 0.001$ for noninferiority). However, all-cause mortality was slightly lower in the exenatide group versus placebo group (HR = 0.86; 95% CI: 0.77–0.97). There was no progression to retinopathy and the drug was well-tolerated overall [89].

Conflicts of Interest

The authors report no multiplicity or conflicts of interest.

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Review Article

Macrovascular Complications in Patients with Diabetes and Prediabetes

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Diabetes is a significant health problem worldwide, and its association with cardiovascular disease (CVD) was reported in several studies. Hyperglycemia and insulin resistance seen in diabetes and prediabetes lead to an increase in reactive oxygen species, which triggers intracellular molecular signaling. The resulting prothrombotic state and increase in inflammatory mediators expedite atherosclerotic changes and the development of macrovascular complications. Individuals with diabetes or prediabetes have a higher risk of developing myocardial infarction, stroke, and peripheral artery disease. However, no significant difference in cardiovascular morbidity has been observed with tight glycemic control despite a reduction in some CVD outcomes, and the risk of adverse outcomes such as hypoglycemia was increased. Recently, some GLP-1 receptor agonists and SGLT-2 inhibitors have been shown to reduce cardiovascular events and mortality. In this review we give an overview of the risk and pathogenesis of cardiovascular disease among diabetic and prediabetic patients, as well as the implication of recent changes in diabetes management.

1. Introduction

Diabetes has been recognized as a global epidemic, with the number of adults with diabetes reaching 422 million and an estimated prevalence of 8.5% worldwide in 2014 [1]. However, the prevalence of diabetes is heterogeneous and varies according to nations. In Arabic countries such as Qatar, it is estimated to be 20.2%, whereas in the United States the prevalence is about 12.3% [2, 3], suggesting a more gravid healthcare burden and more pressing issue.

Diabetes is a leading cause of microvascular complications such as nephropathy and retinopathy. It is also associated with an accelerating atherosclerosis, and type 2 diabetes mellitus (T2DM) is usually not detected until late in the course of cardiovascular disease (CVD). Therefore, many patients are suffering from complications at or shortly after diagnosis. The strong association between diabetes and CVD was observed in multiple studies, independently of other traditional cardiovascular risk factors [4–7]. Being the most common cause of mortality in diabetic patients, CVD mortality accounts for 52% of deaths in T2DM and 44% in type 1 diabetes mellitus (T1DM) [8].

Recently, prediabetic states, characterized by impaired fasting glycaemia (IFG) or impaired glucose tolerance (IGT), have also been shown to be associated with CVD morbidity and mortality [9, 10]. It is therefore important to have a better understanding of the pathophysiology, in order to identify new approach to tackle or prevent the development of macrovascular complications early on. This article attempts to review current understanding of the epidemiology, pathogenesis, and implication of increased CVD risk in diabetic and prediabetic population.

2. Pathogenesis of CVD in Diabetes Mellitus

Hyperglycemia and insulin resistance, among various other factors, are thought to contribute significantly to atherosclerotic changes and the pathogenesis of macrovascular complications in diabetes. Though both are commonly observed in diabetic patients, insulin resistance usually develops years before hyperglycemia becomes clinically significant.

2.1. Insulin Resistance. Obesity plays an important part in the pathogenesis of insulin resistance, which is commonly seen in T2DM patients. By releasing free fatty acids (FFAs) and inflammatory mediators, adipose tissue alters lipid metabolism, increases reactive oxygen species (ROS) production, and increases systemic inflammation [11]. Insulin resistance is related to abnormal function of the glucose transporter type 4 (GLUT-4), the insulin-mediated glucose transporter mainly found in adipose cells and muscle cells. When FFAs bind to Toll-like receptor (TLR), PI3-kinase (PI3K) and Akt activity are downregulated, which reduces expression of GLUT-4 [12], leading to decreased response to insulin binding.

Meanwhile, decreased PI3K and Akt activity also lead to inactivation of endothelial nitric oxide synthase (eNOS), which reduces nitric oxide (NO) production [13]. NO activity is further reduced by increased ROS generation caused directly by obesity and insulin resistance, due to the NO-inactivating effect of ROS. NO is a key molecule in maintaining normal function of endothelial cells. Obesity and insulin resistance induced decrease in NO activity, thus contributing to endothelial dysfunction and subsequent atherosclerotic changes (Figure 1).

In addition to downregulation of PI3-kinase and Akt, the binding of FFAs to TLR also activates nuclear factor NF-κB, which triggers transcription of inflammatory molecules, contributing to insulin resistance and atherosclerosis development [12]. The blockade of NF-κB in a mice model resulted in decrease in systemic oxidative markers, adhesion molecule gene expression, and macrophage infiltration, processes that contribute to atherosclerosis [14], suggesting an important upregulation role of NF-κB in CVD development.

Parallel to atherosclerotic changes, thrombosis also plays an important role in the development of macrovascular complications in diabetes. In physiological setting, insulin inhibits thrombosis and increases fibrinolysis, and insulin resistance creates a prothrombotic state [15]. Lack of insulin also results in calcium accumulation in platelets, which enhances platelets aggregation [16], further contributing to CVD development.

2.2. Hyperglycemia. Hyperglycemia is also involved in the pathogenesis of cardiovascular complication of diabetes. It increases the production of ROS, which inactivates NO [17], leading subsequently to endothelial dysfunction. On the other hand, increased ROS production contributes to CVD by triggering the activation of protein kinase C (PKC). Acting as a group of enzymes that can affect the function of other cellular proteins, PKC has been shown to have an effect on vascular cell growth and apoptosis, permeability, extracellular matrix synthesis, and cytokine production [18]. Activation of PKC results in alteration of vascular homeostasis and predisposition to vascular complications. PKC in turn induces ROS production in vascular cells [19], perpetuating the vicious cycle (Figure 1).

PKC also affects endothelial cells in different molecular aspects, including inactivation of NO and overproduction of vasoconstrictors. As mentioned above, PKC increases production of ROS, which decreases NO availability. At the same time, PKC directly decreases eNOS activity, by inhibiting eNOS gene expression [20]. PKC also induces vasoconstrictor synthesis: the production of endothelin-1 (ET-1), a molecule involved in platelet aggregation and vasoconstriction, is upregulated by PKC activation [18]; PKC enhances activity of cyclooxygenase-2 (COX-2) expression, which increases thromboxane A2 (TXA2) and decreases prostacyclin (PGI_2) production. The combination of reduced NO availability and increased vasoconstrictor production promotes the development of vascular atherosclerotic changes.

Hyperglycemia and PKC activation-induced ROS production causes inflammatory changes in vascular endothelium. With increased ROS level, the nuclear factor NF-κB subunit p65 expression and nuclear translocation are upregulated, leading to increased transcription of genes encoding inflammatory factors [21]. The increased production of inflammatory mediators leads to monocytes adhesion, extravasation, and formation of foam cells, further contributing to the development of atherosclerosis. Chronic hyperglycemia is also responsible for cardiovascular damage through activation of other major biochemical paths including polyol pathway flux, increased formation of advanced glycation end products (AGEs), increased expression of AGEs receptor and its activating ligands, and overactivity of the hexosamine pathway [22].

3. Risk of Coronary Heart Disease among Patients with Diabetes

Diabetes is associated with increased risk of coronary heart disease (CHD). In patients without prior history of myocardial infarction (MI), the 7-year risk of MI is 20.2% and 3.5% for diabetics versus nondiabetics, respectively. Similarly, in patients with a history of MI, the 7-year risk of MI is 45.0% and 18.8% for diabetics and nondiabetics, respectively [22]. The 7-year risk of developing MI in diabetic patients was comparable to the risk of MI in nondiabetic patients who have had a prior MI, which suggests that diabetes contributes significantly to the development of MI and can possibly be considered as a CHD risk equivalent. However, a population study, which included adult residents in Denmark who are 30 years or older, showed that diabetes increased the risk of CHD but not to the extent of a risk equivalent during the 5-year follow-up. In this study, men with diabetes had a hazard ratio (HR) of 2.30 for developing MI, which was lower than the risk of nondiabetic men with a history of prior MI (in whom HR = 3.97). Similar findings were observed for CHD mortality, incidence of total CVD events, and cardiovascular mortality [23]. In a meta-analysis consisting of 13 studies, diabetic patients who did not have a history of MI have a 43%

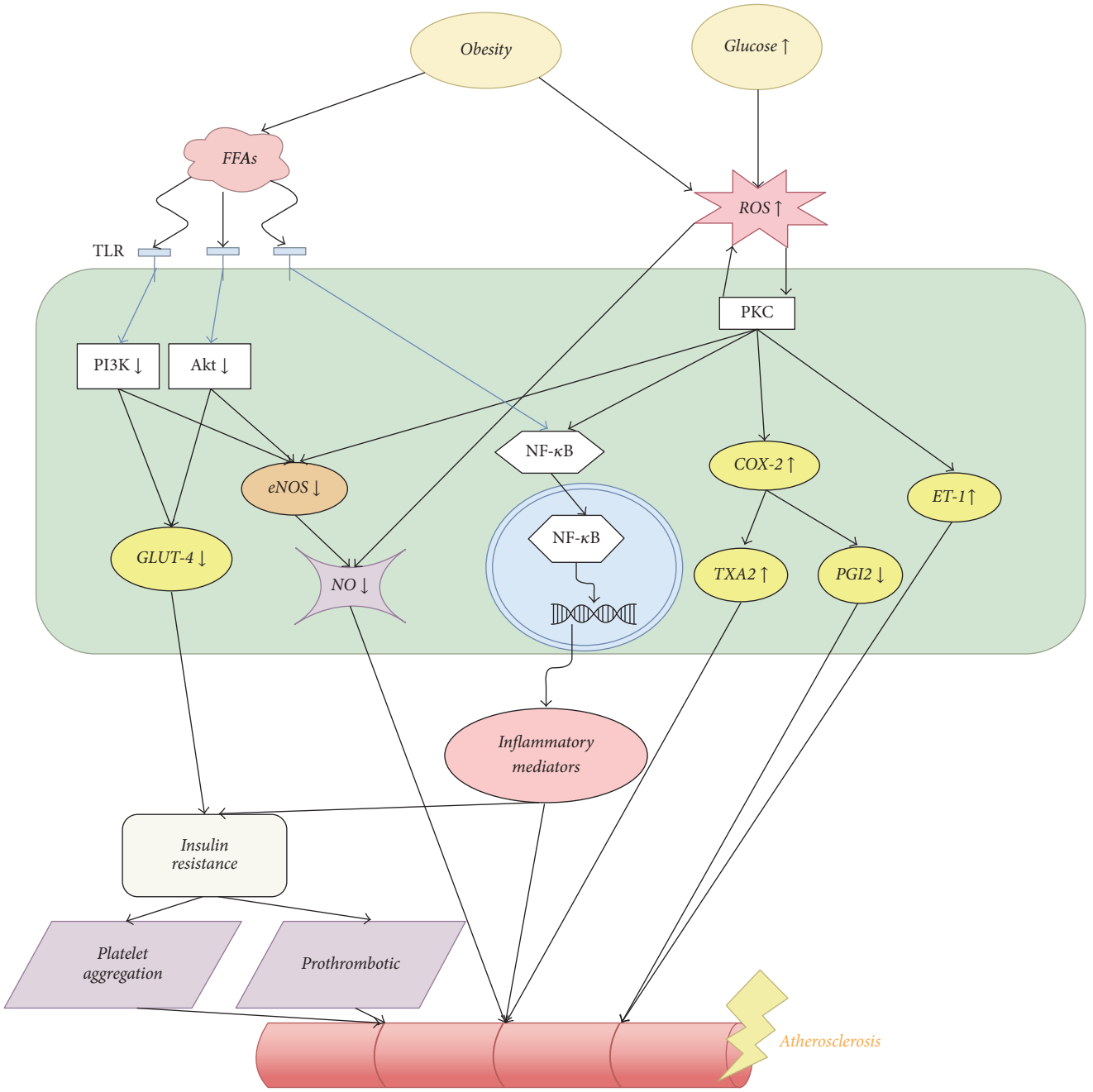

Figure 1: *The effect of insulin resistance and hyperglycemia in CVD pathogenesis*. Insulin resistance is tightly correlated with obesity, which increases FFA and ROS level, both of which contribute to atherosclerotic changes and the development of macrovascular complications. Increased plasma glucose level contributes to increased production of ROS as well, which activates PKCs intracellularly and leads to inflammatory changes and atherosclerosis. FFAs: free fatty acids, ROS: reactive oxygen species, TLR: Toll-like receptor, PI3K: PI3-kinase, PKC: protein kinase C, eNOS: endothelial nitric oxide synthase, NO: nitric oxide, COX-2: cyclooxygenase-2, TXA2: thromboxane A2, PGI_2: prostacyclin, ET-1: endothelin-1.

lower risk of developing CHD compared to nondiabetics with prior history of MI [24].

Diabetes has also a negative impact on the treatment of CHD. When evaluating percutaneous coronary intervention performed in patients with ST-elevation MI, those with diabetes had a higher 3-year risk of target lesion revascularization, MI recurrence, and all-cause mortality, as compared to those without diabetes [25]. The analysis of patients treated with drug-eluting stent after MI showed that diabetes is more prevalent in patients who developed stent thrombosis than in those who did not [26]. Diabetic patients are 1.8 times more likely to develop stent thrombosis than nondiabetic patients 1 year after stenting [27]. In terms of coronary artery bypass graft (CABG) surgery, patients with diabetes

had a significantly higher operative mortality, with a relative risk of 1.67 compared to nondiabetics [28]. Interestingly, by achieving intensive antiplatelet effect with prasugrel, patients with diabetes have a more significant reduction of MI incidence compared to those without diabetes [29]. This finding may suggest a significant role of platelet activation and aggregation in the development of CHD in diabetes.

4. Risk of Stroke among Diabetics

Other than CHD, diabetes also increases the risk of stroke. The INTERSTROKE study, which is a case-control study that recruited patients who developed acute stroke and those without a stroke history in 22 countries, demonstrated a 35% increase in stroke risk in patients with self-reported history of diabetes [30]. In a meta-analysis with 102 prospective studies, diabetic patients had a 2.3-time higher risk of developing ischemic stroke and a 1.6-time higher risk of developing hemorrhagic stroke compared to nondiabetics [31].

Diabetes is also correlated with a worse outcome and more disability after stroke. Among patients admitted for acute stroke, diabetes was associated with a higher risk of death or functional dependency (characterized by modified Rankin Scale of 3–6) [32]. The Australian Stroke Unit Registry demonstrated a worse functional outcome 3 months after acute stroke in patients with diabetes compared to those without [33]. Patients with impaired fasting glycaemia also showed a poor functional outcome and a lower rate of discharge to home after acute stroke [34].

Stroke recurrence is also affected by diabetes. The Dutch TIA trial, which studied patients who developed minor ischemic stroke or transient ischemic attacks (TIAs), showed a 2.10-time higher risk of developing nonfatal stroke among diabetic patients compared to nondiabetic subjects [35].

5. Risk of Peripheral Arterial Disease among Diabetics

Peripheral arterial disease (PAD) is a common macrovascular complication in patients with diabetes. The German Epidemiological Trial on Ankle Brachial Index (GETABI) study demonstrated that among patients aged 65 or older, diabetic patients have a 2-fold higher rate of PAD (defined as ABI < 0.9), as well as a 2.5-fold higher risk of intermittent claudication [36]. In patients diagnosed with PAD, the risk of developing an ischemic ulceration is increased by more than 20% in 10 years, with a 3-fold higher likelihood among diabetics. Moreover, 30% of the patients were found to have ischemic rest pain during the follow-up, and diabetes increased the risk by 1.8-fold [37].

ABI is useful in identifying PAD and quantifying PAD severity. The value 0.9 has been used as a cut-off for signs of arterial occlusion. However, diabetic patients may have certain degrees of arterial occlusion at a higher ABI value, which results in underdiagnosis of PAD in this population [38]. A study demonstrated that the cut-off value with the highest sensitivity and specificity for diabetic patients is somewhere between 1.0 and 1.1 [39]. The sensitivity of ABI is significantly limited in diabetic patients compared to nondiabetics, which could be partially explained by the arterial stiffness resulting from medial artery calcification [40]. Calcification causes the vessels to be poorly compressible and increases ABI. In fact, in diabetic patients, particularly those with impaired renal function, ABI > 1.4 is also suggestive of PAD [41].

Diabetes is also associated with worse revascularization outcomes [42]. In addition to the higher risk of limb loss, there is also a significant increase in cardiovascular event rates in patients with PAD. Decreased ABI has been shown to be an independent risk factor for CVD event, cardiovascular mortality, and overall mortality. In a retrospective follow-up of over 450 patients with T2DM, ABI less than 0.9 was associated with a significant increase in the primary composite endpoint of major cardiovascular events and in the secondary endpoint of all-cause mortality, compared to ABI equal to or higher than 0.9 [43]. In a similar prospective cohort of 3000 Japanese individuals, a low ABI was independently associated with a higher incidence of cardiovascular events and mortality in patients with and without T2DM [44].

6. Relation of CVD with Prediabetes

Disturbed glucose metabolism plays a major role in atherosclerosis and CVD. Cumulative data are suggesting that increased plasma glucose level is a risk factor for CVD regardless of the presence of diabetes. A prediabetic state could be defined by IFG (fasting glucose level of 5.6–6.9 mmol/l), IGT (2-hour postcharge glucose of 7.8–11.0 mmol/l), and/or HbA_{1c} level of 5.7%–6.4% [45].

Compared to those with a fasting glucose level of 3.90–5.59 mmol/l, those with a level higher than 5.60 mmol/l (i.e., prediabetic or diabetic) have an increased risk of developing CHD [31]. In the Heart Outcomes Prevention Evaluation (HOPE) study, the risk of cardiovascular events (MI, stroke, and cardiovascular death) in the following 4.5 years increases by almost 9% with every 1 mmol/l increase in fasting glucose. Every 1% increase in HbA_{1c} was also correlated with a higher risk of cardiovascular outcomes, with a relative risk of 1.07. These relationships were independent of other cardiovascular risk factors (age, sex, blood pressure, and hyperlipidemia) and remained significant after adjustment for diabetic status [9]. Similarly, the Diabetes Epidemiology: Collaborative analysis Of Diagnostic criteria in Europe (DECODE) study showed a correlation between fasting plasma glucose and CVD-associated mortality, independently of diabetic status. The relationship between fasting plasma glucose and CVD-associated mortality seemed to be "J-shaped" curve, with no threshold effect observed at high glucose level [46].

However, the relation between CVD and fasting glucose level in the prediabetic range is not consistent among studies. The Hoorn study, a cohort study in the Dutch population, demonstrated that fasting glucose levels are correlated with cardiovascular mortality in the diabetic range, but not in the prediabetic range. However, the same study showed that postprandial glucose level and HbA_{1c} levels predict an increase in the 8-year risk of cardiovascular mortality, in both diabetics and nondiabetics [47].

IGT and HbA_{1c} appear to correlate more with CVD risk than IFG. The Funagata Diabetes Study, a cohort study in Japanese population, observed a correlation between CVD and IGT, but not with IFG [48]. The Framingham Offspring study made similar observations [49]. When analyzed separately, CVD incidence during the 4-year follow-up correlated with fasting glucose, glucose tolerance, and HbA1c, with a relative risk of 1.13 for every 0.7 mmol/l increase in fasting glucose, 1.26 for every 2.1 mmol/l increase in postprandial glucose, and 1.24 for every 0.7% increase in HbA1c. When analyzed in the same model, fasting glucose had a much weaker effect, while postprandial glucose still significantly increases CVD risk. In a meta-analysis comprised of 53 cohort studies, patients with prediabetic states were found to be at an increased risk for CVD, CHD, and stroke. Patients with IGT had a higher risk compared to those with IFG [50]. In a prospective study of nondiabetic patients admitted for MI with a blood glucose level < 11.1 mmol/l, 35% of the patients were found to have IGT at discharge. At 3-month follow-up, 31% fulfilled the criteria of diabetes [51]. Another study with a larger patient population reached similar conclusions. Among the patients admitted for an acute coronary syndrome, 36% were found to have IGT and 22% previously undiagnosed diabetes [52].

7. Recent Trend

During the last decade, with better recognition of the adverse effect imposed by diabetes and availability of novel pharmacological reagents, we have observed better control of glycemia, HbA_{1c}, blood pressure, and lipid profile in diabetic patients. Meanwhile, the risk of CVD has significantly decreased. With the UK Prospective Diabetes Study (UKPDS) algorithms, the estimated 10-year risk for CHD among diabetic patients was 21.1% in the period of 1999-2000, which has decreased to 16.4% in 2007-2008 [53]. In the US adult population, the CVD-associated mortality rate among diabetic patients decreased by 40% from 1997-1998 to 2003-2004, while the diabetes-associated excess CVD-associated mortality rate was reduced by 60% [54]. A similar trend was observed with the Swedish population, where modifiable CHD risk among diabetics decreased from 37.7% in 2003 to 19.1% in 2008 [55].

The prevalence and outcome of stroke have also improved over the past few decades. Between 1992 and 2002, the incidents of first CVD—including CHD and ischemic stroke—decreased in patients with diabetes in Finland [56]. Another study showed that although mortality after the first ischemic stroke is higher in patients with diabetes than nondiabetics, the mortality rate among diabetes has declined over the study period of 1988–2002 [57].

Unlike CHD and stroke, the prevalence of PAD among diabetics was not significantly different with intensive treatment of diabetes in addition to current standard diabetic care [58]. The incidence of PAD varied among studies. A study in Queensland demonstrated that between 2005 and 2010, the incidence of hospitalization related to PAD among diabetics has decreased by 43%, and the incidence of amputation has decreased by 40% [59]. However, a Spanish study showed a significant increase in lower limb amputation rate in patients with T2DM from 2001 to 2008 [60]. The discrepancy could be due to different quality of care for diabetes-related foot conditions, among other factors. Data from 84 hospitals in Los Angeles showed significant variability in rates of lower extremity amputation between different types of hospitals [61].

8. The Effect of Glycemic Control on Macrovascular Complications

The association of diabetes and prediabetes with CVD and recent changes in CVD prevalence among diabetes suggests the possibility of preventing CVD development by better controlling diabetes. Considering the role of hyperglycemia in CVD pathogenesis, tight glycemic control seems to be a reasonable approach for many decades, which has been investigated by multiple clinical trials (Table 1).

The Action to Control Cardiovascular Risk in Diabetes (ACCORD) study randomized diabetic patients to an intensive therapy group with targeting HbA_{1c} < 6.0% and a group receiving standard therapy with targeting HbA_{1c} 7.0–7.9%. In 1 year, the patients with intensive glycemic control had a reduced incidence of CVD, but not statistically significant reduction of macrovascular events. Meanwhile, mortality, incidence of hypoglycemic events, and weight gain were significantly higher in the group receiving intensive glycemic control compared to that receiving standard therapy [62]. Similarly, the Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE) trial compared standard treatment with intensive glycemic control, which involved the use of gliclazide and other hypoglycemic agents as needed. 11,140 patients who have been diagnosed with T2DM were randomly assigned to two groups. The intensive treatment group achieved an average HbA_{1c} of 6.5% compared to 7.3% in the control group in median 5-year follow-up. The study showed no significant difference in the incidence of macrovascular complications, cardiovascular mortality, and overall mortality between the two groups. However, the incidence of severe hypoglycemia was higher in the intensive glycemic control group, with a hazard ratio of 1.86 [63]. Comparable outcomes were observed in the Veterans Affairs Diabetes Trial (VADT). The trial recruited 1791 military veterans, whose T2DM was not optimally controlled. The patients were randomized to standard treatment group and intensive therapy group. At a median 5.6-year follow-up, there was no significant difference in major CVD, CVD-associated mortality, and other macrovascular complications. The rate of adverse events, mainly hypoglycemia, was significantly higher in the intensive therapy group compared to the standard treatment group [64].

Furthermore, two meta-analyses demonstrated that intensive glucose control reduced the incidence of cardiovascular events, particularly nonfatal MI. The CVD-associated mortality and overall mortality, however, were not significantly different, and the risk of hypoglycemia is higher in the intensive therapy group than the standard treatment group [65, 66]. The observations from the above-mentioned

Table 1: *Intensive glycemic control and cardiovascular events in type II diabetes*. There was comparable CVD risk in the intensive glycemic control group and the standard therapy group. However, the risk of hypoglycemia is significantly higher in the groups with intensive control therapy.

Clinical trial	ACCORD		ADVANCE		VADT	
Sample size	10,251		11,140		1,791	
Median follow-up	3.5 years		5 years		5.6 years	
Treatment group	Intensive control	Standard therapy	Intensive control	Standard therapy	Intensive control	Standard therapy
Mean HbA1c	6.7%	7.5%	6.5%	7.3%	6.9%	8.4%
CVD event	6.9% ($p = 0.16$)	7.2%	10.0% ($p = 0.32$)	10.6%	30.0% ($p = 0.14$)	34.0%
CVD mortality	2.6% ($p = 0.02$)	1.8%	4.5% ($p = 0.12$)	5.2%	4.5% ($p = 0.29$)	3.7%
All-cause mortality	5.0% ($p = 0.04$)	4.0%	8.9% ($p = 0.28$)	9.6%	11.4% ($p = 0.62$)	10.6%
Hypoglycemia	10.5% ($p < 0.001$)	3.5%	2.7% ($p < 0.001$)	1.5%	1333 episodes ($p < 0.001$)	383 episodes

trials lead to the conclusion that intensive glycemic control alone is not enough to prevent macrovascular complications. Nevertheless, an approach based on multiple risk control of macrovascular disease offers a reduction in mortality and macro- and microvascular events as demonstrated by the STENO-2 study [67, 68].

Insulin resistance also plays a role in CVD pathogenesis and is therefore a possible therapeutic target. In diabetic patients with established atherothrombosis, metformin treatment reduced the all-cause mortality rate from 9.8% to 6.3% [69]. In the UKPDS study, patients who had been recently diagnosed with diabetes were randomized to a dietary restriction group or an intensive treatment group. The intensive treatment group involves the use of sulfonylurea, insulin, or metformin. Patients on metformin had less reduction of HbA_{1c} levels compared to those on sulfonylurea or insulin. However, a greater reduction of MI and overall mortality was observed in the metformin group compared to the sulfonylurea group at 5-year follow-up [70].

However, this neutral trend was recently reversed with new antidiabetic medications such as the GLP-1 receptor agonists and the SGLT-2 inhibitors. It is worth noting that those studies were not designed to test whether an intensive glycemic treatment would reduce cardiovascular events compared to a conventional one, but rather to assess cardiovascular safety. In the Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial, liraglutide was superior to placebo in reducing major cardiovascular events when added to the conventional treatment of individuals with T2DM at high cardiovascular risk [71]. A similar decrease in major events was also reported in the Semaglutide and Cardiovascular Outcomes in Patients with Type 2 Diabetes (SUSTAIN-6) trial despite a relatively short follow-up period (mean: 2.1 years) and an $HbA_{1c} > 7.5\%$ at the end of the trial in both groups [72]. However, 2 other GLP-1 agonist trials did not show a reduction in cardiovascular events: the Evaluation of Lixisenatide in Acute Coronary Syndrome *(ELIXA)* [73], which randomized T2DM patients post-MI to lixisenatide or placebo, and the recently published Exenatide Study of Cardiovascular Event Lowering (EXSCEL) trial that failed to demonstrate a cardiovascular benefit of weekly injections of exenatide [74]. The Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes (EMPA-REG) trial is the first to assess cardiovascular outcomes of an SGLT-2 inhibitor in T2DM patients. Interestingly, empagliflozin decreased cardiovascular mortality and was also associated with a 35% risk reduction of hospitalization for heart failure [75]. In the recently published Canagliflozin and Cardiovascular and Renal Events in Type 2 Diabetes (CANVAS) trial, patients with T2DM and high cardiovascular risk experienced less cardiovascular events during the mean of 3.6 years [76]. Other SGLT-2 inhibitors and GLP-1 receptor agonists' trials are expected to be presented within the coming years.

9. Conclusion

In summary, both diabetes and prediabetes predispose patients to the development of macrovascular complications of diabetes, through complex molecular pathways that involve hyperglycemia and insulin resistance. While intensive glycemic control alone might not reduce mortality and major cardiovascular events, a global approach consisting of life-style modifications, decreasing hyperglycemia, and treating cardiovascular risk factors associated with diabetes is beneficial to the cardiovascular risk profile of those patients; hence, the target of blood glucose control should be tailored to the individual patients. In recent years, a new hope has risen with the new class of antidiabetic agents such as SGLT-2 inhibitors and GLP-1 receptor agonists to decrease mortality in patients with T2DM without increasing the risk of hypoglycemia.

Disclosure

All of the below-mentioned funding sources did not have a role in the study's concept, analysis, or manuscript writing.

Conflicts of Interest

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

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Review Article

New Antihyperglycemic Drugs and Heart Failure: Synopsis of Basic and Clinical Data

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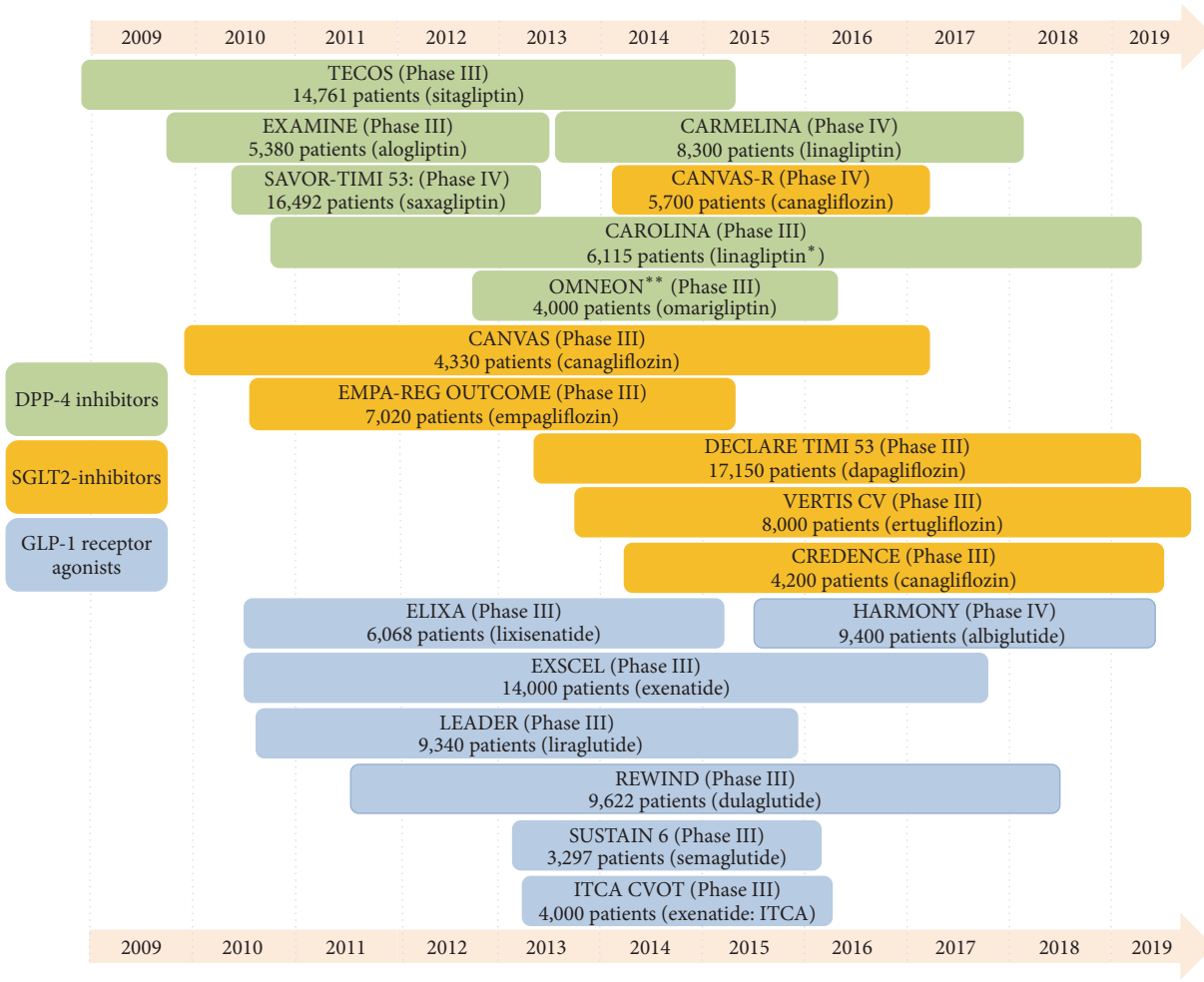
The assessment of the cardiovascular safety profile of any newly developed antihyperglycemic drug is mandatory before registration, as a meta-analysis raised alarm describing a significant increase in myocardial infarction with the thiazolidinedione rosiglitazone. The first results from completed cardiovascular outcome trials are already available: TECOS, SAVOR-TIMI, and EXAMINE investigated dipeptidyl peptidase 4 (DPP-4) inhibitors, ELIXA, LEADER, and SUSTAIN-6 investigated glucagon-like peptide 1 (GLP-1) receptor agonists, and EMPA-REG OUTCOME and CANVAS investigated sodium-dependent glucose transporter 2 (SGLT-2) inhibitors. LEADER, SUSTAIN-6, EMPA-REG OUTCOME, and CANVAS showed potential beneficial results, while the SAVOR-TIMI trial had an increased rate of hospitalization for heart failure. Meanwhile, the same drugs are investigated in preclinical experiments mainly using various animal models, which aim to find interactions and elucidate the underlying downstream mechanisms between the antihyperglycemic drugs and the cardiovascular system. Yet the direct link for observed effects, especially for DPP-4 and SGLT-2 inhibitors, is still unknown. Further inquiry into these mechanisms is crucial for the interpretation of the clinical trials' outcome and, vice versa, the clinical trials provide hints for an involvement of the cardiovascular system. The synopsis of preclinical and clinical data is essential for a detailed understanding of benefits and risks of new antihyperglycemic drugs.

1. Introduction

Throughout the last decade, demonstration of glucose lowering efficacy was the primary basis for the approval of antihyperglycemic drugs. However, increasing concerns about the cardiovascular safety profile of already approved glucose lowering drugs or drugs under consideration for approval have emerged. In 2007, Nissen and Wolski published their meta-analysis describing a relative 43% increase in myocardial infarction with the use of thiazolidinedione rosiglitazone [1]. The Food and Drug Administration (FDA) and the European Medicines Agency (EMA) responded by mandating the demonstration of the cardiovascular safety profile of novel antihyperglycemic drugs, requiring a cardiovascular outcome trial [2]. This novel regulation has changed the landscape for clinical trials in the field of diabetes significantly and since 2008 more than 160,000 patients have been enrolled in cardiovascular outcome trials (Figure 1) [3]. Augmenting data on potential cardiovascular side effects of antidiabetic drugs is very valuable since millions of people are treated over many years. In most of these patients, multiple cardiovascular risk factors are commonly present, so lowering the risk for macrovascular complications is one of the major tasks in current multifactorial diabetes management. Over the last years besides the classical primary ischemic endpoints, heart failure has emerged as an increasingly important endpoint in



(i) * Linagliptin versus glimepiride
(ii) ** Study terminated for business reasons

Figure 1: Timeline of already completed and still running cardiovascular safety trials. Green: DPP-4 inhibitors, orange: SGLT-2 inhibitors, and blue: GLP-1 receptor agonists. Name and number of planned included patients are given. All trials tested drugs versus placebo except the CAROLINA trail (linagliptin versus glimepiride).

diabetes outcome trials. Diabetes is a major risk factor for the development of heart failure [4], with approximately 22% of subjects with type 2 diabetes at an age above 65 years having a heart failure diagnosis [5].

Since 2013, eight of the FDA and EMA mandated trials have reported their results. There is no doubt that major cardiovascular events (MACE), death, and heart failure are indeed robust clinical endpoints; however, some of the results such as the potential heart failure signal for the dipeptidyl peptidase 4 (DPP-4) inhibitor saxagliptin in SAVOR-TIMI 53 or the pronounced cardiovascular benefit of the sodium-dependent glucose transporter 2 (SGLT-2) inhibitors empagliflozin and canagliflozin were rather surprising. Interestingly, there is little mechanistic insight to derive from these outcome trial data explaining cardiovascular harm or benefit. To sufficiently power these outcome trials while keeping the number of subjects and follow-up duration within acceptable limits, patients with diabetes and high cardiovascular risk or previously diagnosed atherosclerotic disease are randomized in these trials. However, the majority of patients with diabetes in routine care do not have a cardiovascular risk as high as represented by these trials [6].

This must be kept in mind, especially when findings from these outcome trials are extrapolated to patients with low cardiovascular risk. Performing outcome trials in the primary prevention setting would be important to inform future diabetes treatment, although this is a challenging task: given a MACE rate of approximately one-third as compared to subjects in the secondary prevention setting, trials in low cardiovascular risk patients would need to last longer, include more subjects, or combine both approaches, leading to a significant increase in the costs for such trials. Therefore, the synopsis of outcome data and results of basic research on cell and tissue level in models with elevated or not-elevated

cardiovascular risk are of relevance and discussed in this review.

2. Diabetic Heart

Heart failure in diabetes represents a multifactorial problem resulting from a variety of cardiotoxic factors, such as coronary artery disease, hypertension, and direct harmful effects of glucose on the myocardium [7]. Besides well characterized macrovascular effects leading to coronary heart disease and corresponding clinical events, there is increasing data suggesting that there are direct associations between diabetes and heart failure. A 2-fold higher risk of heart failure in male diabetics and a 5-fold increase in risk in female patients with diabetes have already been demonstrated in the Framingham study [8] and this association is of particular importance in younger patients [5]. The underlying mechanisms include but are not limited to increased interstitial and perivascular fibrosis. This histological pattern was considered the basis for the term "diabetic cardiomyopathy" in the early 1970s [9]. This type of fibrosis is independent of coronary artery disease or hypertension [10]. Nonetheless, diabetic cardiomyopathy remains only moderately understood. Advanced glycation end products (AGE) [11] and increased content crosslinking of collagen seem to play a significant role [12–14]. Besides histological findings, calcium homeostasis is probably affected directly as indicated by lower activity levels of the sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) in diabetic hearts [15]. Moreover, SERCA2a is a major regulator of glucose transport in the healthy and diabetic heart via calcium mediated glucose transporter (GLUT) type 4 translocation [16].

There is robust evidence that metabolic abnormalities underlie the impaired myocardial function in heart failure. Metabolic parameters such as the adenosine triphosphate to phosphocreatine ratio (ATP/PCr) have been shown to predict outcome even better than left ventricular ejection fraction (LV-EF) or the clinical NYHA class [17]. In addition, changes in myocardial metabolism show direct and acute effects on mechanical performance and this effect seems to be of particular importance in human myocardium. Insulin administration itself exerts positive inotropic effects in human ventricular myocardium via Ca^{2+}-dependent and Ca^{2+}-independent mechanisms. Both mechanisms raise the load of the sarcoplasmic reticulum (SR) resulting in an increase of systolic Ca^{2+}-transients as well as an increase in myofilament sensitivity [18]. The metabolic changes upon insulin administration could be traced back to altered GLUT-4 translocation and SGLT-1 activation [19, 20]. Additionally, insulin administration does not only result in acute functional effects, but also triggers various approaches modifying the energy substrate metabolism via an increased rate of pyruvate supply, as shown in vitro as well as in vivo [21, 22].

Heart failure and diabetes interact bidirectionally. Besides an HbA1c dependent increased risk of developing heart failure in patients with diabetes mellitus, the prevalence of diabetes in heart failure patients is known to increase markedly over time (3.8% per year) [23, 24]. Experimental data provides insight into substance-specific effects of glucose lowering therapy in heart failure. So far, with respect to the single classes of antidiabetic drugs and the related individual substances, the amount and quality of available experimental data are heterogeneous.

3. DPP-4 Inhibitors

While sitagliptin, alogliptin, and saxagliptin were shown to be safe for the cardiovascular system in terms of the MACE, cardiovascular death, and heart failure endpoints, the SAVOR-TIMI 53 trial showed a rather surprising signal for an increased risk for hospitalization of heart failure in the saxagliptin group, especially in the subgroups of impaired renal function and preexisting heart failure [25]. A similar trend could be observed for alogliptin in the EXAMINE trial (EXAMINE), albeit not statistically significant. In contrast, TECOS did not show an increased rate for heart failure hospitalizations after sitagliptin administration, suggesting a potential difference between members of the DPP-4 inhibitor class. The cardiovascular outcome trials CARMELINA and CAROLINA (both for linagliptin) are still running and results are expected in 2018 and 2019, respectively. Recent meta-analyses including the finished major and many smaller cardiovascular safety studies for DPP-4 inhibitors have different conclusions, ranging from no increased risk for the hospitalization of heart failure after DPP4 inhibitor use [26] to an increased risk [27].

However, studies that examine the potential pleiotropic and nonglycemic effects of DPP-4 inhibitors on various cells and tissues may help to understand and interpret the difference in the observed cardiovascular side effects in some of the clinical trials. Recently, many reviews have tried to clarify the effects caused by DPP-4 inhibitors. They interact strongly with the heart, vascular system, kidney, liver, neuroendocrine system, immune system, and hematopoietic system affecting hormones or second messengers like brain natriuretic peptide (BNP), substance P, activation of chemokine and cytokine pathways, intracellular calcium concentrations, and the release of nitric oxide (NO) shown in different animal models in vivo and ex vivo [28–32]. Interactions of DPP-4 inhibitors with the cardiovascular system and cardiomyocytes were successfully revealed, yet a direct link between DPP-4 inhibitors and its effects on cardiac contractility and/or electrophysiological function is still unknown, and the corresponding downstream mechanisms have yet to be determined. Therefore, studies that explored effects of DPP-4 inhibitors on cardiovascular system are of particular interest.

For saxagliptin, overwhelming potential beneficial effects are reported in literature: it reduces the damage of blood vessels via the amelioration of the availability of NO and the reduction of cyclooxygenase-1-action derived vasoconstriction caused by induced type-2 diabetes mellitus in mice [33] and, similarly, leads to a restoration of damaged mitochondrial vascular function in diabetic rats [34]. Additionally, a reduction of blood pressure by increasing the bioavailability of NO in spontaneous hypertensive rats [35] and an improvement of cardiac function after myocardial infarction independent of glucose lowering [36] could be demonstrated

in diabetic rats. One study clarified that saxagliptin alters the cGMP-PKG-PDE5 axis in a swine model that mimicked heart failure with preserved ejection fraction (HFpEF) by aortic banding thus preventing left ventricular damage and improving left ventricular systolic and diastolic function [37]. Another study that explored the effects of saxagliptin on human multicellular myocardium and guinea pig ventricular cardiomyocytes revealed a negative inotropic potential, the prolongation of the action potential duration, and the occurrence of arrhythmias although the exact mechanism has not yet been determined [38].

Similar effects are reported for sitagliptin in diabetic rats. Sitagliptin improved endothelial function [39] and attenuated cardiac remodeling without affecting systolic function after myocardial infarction [40] while, in normoglycemic rats with induced myocardial infarction, sitagliptin prevented fatal arrhythmias by attenuating GIP-dependent resistin signaling [41] and in a PKA-dependent pathway [42]. Moreover, sitagliptin attenuated changes in the electrophysiological function in hypertensive rats [43] and counteracted induced HFpEF by improving the diastolic function, decreasing the generation of reactive oxygen species, and reducing proinflammatory biomarkers in the myocardium thus lowering mortality [44, 45]. Similarly, one study proved the reduction of parameters of diastolic dysfunction and myocardial stiffness via the cGMP-PKG pathway after sitagliptin administration in obese diabetic mice [46].

Alogliptin could restore cardiac remodeling and prevent apoptosis via a cAMP-Epac1 dependent and protein PKA-independent mechanism in a model of ventricular pressure overload [47] and inhibited inflammation in arteries that sustained damage by high LDL concentrations [48] in mice. The reported potential beneficial effects might also be present in humans; one trial with a small number of participants showed increased coronary flow reserve and improved left ventricular ejection fraction in patients with type-2 diabetes and coronary artery disease within three months of alogliptin use [49].

For vildagliptin, conflicting results are reported: one study failed to show potential protective effects on cardiac function after myocardial infarction which thereby followed cardiac remodeling despite increased levels of active glucagon-like peptide 1 (GLP-1) in rats [50]. In contrast, other studies suggested that vildagliptin might reduce infarct size and preserve left ventricular ejection fraction by reducing reactive oxygen species in a rat model of ischemia/reperfusion [51] and preventing hypertrophy of the left ventricle after continuous infusion of isoproterenol in rats by the inhibition of inflammatory markers [52]. Additionally, vildagliptin exerts effects via NO and the endothelial NO-synthase (eNOS) leading to an improved vascularization in a mouse model with surgical induced ischemia [53]. Focusing on the cardiovascular system, vildagliptin seems to exert similar effects as sitagliptin [54]. However, no large cardiovascular outcome trial for vildagliptin is being performed.

Finally, linagliptin improves diastolic function in a model of HFpEF in obese rats via an elevated expression of eNOS and improved SERCA2a activity [55]. The effect on eNOS availability could be demonstrated in nonobese mice as well [56]. Linagliptin also reduced angiotensin and glucose induced collagen formation in cardiac fibroblasts of mice by an anti-inflammatory mechanism (via NFkB) [57].

4. GLP-1 Receptor Agonists

The first cardiovascular outcome trial on glucagon-like peptide-1 receptor agonists was the ELIXA trial, which was designed to assess the effects of lixisenatide on the cardiovascular outcome in patients with type-2 diabetes mellitus who had an acute coronary event within 180 days of screening. For the primary composite endpoint (cardiovascular death, myocardial infarction, and stroke), as well as for hospitalization for heart failure, no significant difference was observed between the treatment and placebo group [58]. The LEADER trial assessed the cardiovascular safety of liraglutide in patients with type-2 diabetes mellitus and a HbA1c $\geq$ 7%. Of the total enrolled subjects, 81.3% had preexisting cardiovascular diseases. Liraglutide significantly reduced the rate of the first occurrence of the primary endpoint (cardiovascular death, nonfatal myocardial infarction, or nonfatal stroke) and all-cause mortality. The rates of nonfatal stroke, myocardial infarction, and hospitalization for heart failure were nonsignificantly lower in the liraglutide group compared to the placebo group [59]. In the SUSTAIN-6 trial (semaglutide) patients with type-2 diabetes mellitus and established cardiovascular diseases, chronic heart failure, or chronic kidney disease, or $\geq$60 years with at least one cardiovascular risk factor, were enrolled. Semaglutide significantly reduced the risk for the primary endpoint (first occurrence of cardiovascular death, nonfatal myocardial infarction, or nonfatal stroke). The protective effect of semaglutide on composite endpoints seems to be mainly driven by the reduction of nonfatal stroke [60]. The results of LEADER and SUSTAIN-6 continue to hold promise that GLP-1 receptor agonists might improve CV morbidity in patients with type-2 diabetes mellitus. However, we do not yet fully understand the reasons for the diverging results in the currently published trials. Differences in the duration of action (short acting substances such as lixisenatide versus longer acting drugs like liraglutide or semaglutide) or differences within the amino acid sequences of the peptides are currently being discussed. Further insight will be gained from the imminent presentation of the EXSCEL trail [61].

GLP-1 is an incretin peptide hormone primarily synthesized by intestinal L cells [62]. It is released into the circulation in response to food intake, leading to glucose-dependent insulin release and glucagon suppression. GLP-1(7–36)NH_2, with a half-life of 2 minutes, is the primary active isoform that is rapidly degraded by DPP-4 to GLP-1(9–36)NH_2 [63], a GLP-1 receptor antagonist [64]. Besides increased insulin secretion, GLP-1 receptor activation leads to an inhibition of gastric and small bowel motility, reduces appetite, and subsequently leads to weight loss [65]. In addition, human data suggests that this drug class improves cardiac function in patients with congestive heart failure, ameliorates endothelial dysfunction, and reduces the infarct size after ST-segment-elevation myocardial infarction [66–69]

The GLP-1 receptor is a seven transmembrane, G protein-coupled receptor (GPCR), and is positively coupled to adenylate cyclase through $G\alpha_s$-containing G proteins, which catalyze the conversion of ATP to cAMP. Increased cytosolic cAMP leads to activation of second messenger pathways including PKA, Epac2, and ERK-1/2 [70]. Beneficial effects of GLP-1 receptor agonists have been attributed to direct action on myocardium, with the majority of these effects reported in ventricular cardiomyocytes. However, there are conflicting reports regarding GLP-1 receptor expression in cardiac tissue. Recent studies in mice and rats revealed that the GLP-1 receptor is exclusively localized in atrial cardiomyocytes [71–73]. Wallner et al. reported GLP-1 receptor expression in human right and left ventricular myocardium, although the expression levels were significantly lower compared to right atrial tissue [74]. This discrepancy between human and rodent tissue could be explained by species-related differences, such as those that exist for the SGLT, which is expressed in human myocardium but is undetectable in the myocardium of most species [20].

A recent study in normo- and hypertensive mice suggested that GLP-1 receptor activation in atrial cardiomyocytes increased cAMP levels, promoted Epac2 translocation to the membrane, and increased ANP secretion [71]. Epac2 functions in a PKA-independent manner and, therefore, represents a novel mechanism for governing signaling specificity within the cAMP cascade [75]. A recent study reported significant Epac2 translocation from the cytosol to the cell membrane after GLP-1 receptor activation in human atrial myocardium [74]. Epac2 activation increases phosphorylation of cardiac troponin I (cTnI) in a PKC-dependent manner resulting in increased myofilament Ca^{2+} sensitivity and contractility [76]. GLP-1 receptor agonists significantly increased developed force in human atrial trabeculae, whereas Exendin(9–39)NH_2, a GLP-1 receptor antagonist, and H-89, a PKA inhibitor, blunted the inotropic effect of exenatide. In addition, exenatide (a synthetic GLP-1 receptor agonist that is resistant to the degradation by DPP4) increased PKA-dependent phosphorylation of phospholamban (PLB) and GLUT-1 translocation, but not GLUT-4 translocation [74]. β-Arrestin signaling downstream of GLP-1 receptor activation is another potential mechanism to increase cardiac contractility. β-arrestin, which is well-known for contributing to the termination of GPCR signaling [77], might regulate cardiac function and increase cardiac contractility via β-arrestin-mediated processes [78–80]. Novel "biased ligands" that selectively recruit β-arrestin independent of G protein-mediated signaling have been described for the angiotensin II Type 1A receptor (AT1AR) [78] and the β1-adrenergic receptor (β1AR) [80]. However, Wallner et al. showed that ß-arrestin signaling downstream of GLP-1 receptor activation does not contribute to the positive inotropic effect in human atrial myocardium [74].

5. SGLT-2 Inhibitors

In 2015, the EMPA-REG-OUTCOME trial demonstrated a significant reduction in MACE and all-cause mortality in subjects treated with the SGLT-2 inhibitor empagliflozin [81]. Moreover, this landmark trial showed a 35% relative reduction in the rate of heart failure hospitalization in the empagliflozin group, an effect occurring very quickly after initiating treatment. These findings on MACE and heart failure hospitalization were confirmed in the recently published data from the CANVAS program with canagliflozin [82]. However, cardiovascular and all-cause mortality were not significantly reduced by canagliflozin, in contrast to empagliflozin. Currently, several hypotheses are being discussed for the findings in the SGLT-2 inhibitor trials. These include hemodynamic changes and increased hematocrit that are caused by a diuretic effect or changes in the cardiac fuel metabolism by an improved uptake of β-hydroxybutyrate under conditions of persistent hyperketonemia, all induced by SGLT-2 inhibitors. Particularly ischemic and therefore endangered myocardium may benefit from these effects [83, 84].

For SGLT-2 inhibitors, the most recent class of antidiabetic drugs established for clinical use, there is little data on cardiovascular side effects in animal models or in vitro settings available. This may be a consequence of the fact that the SGLT-2 receptor is not expressed in myocardial tissue [20, 81, 85]. Mechanistically, cardiovascular side effects of SGLT-2 inhibitors could occur either via unselective binding of compounds to SGLT-1, which is not the case for most of the members of this drug class, or via receptor independent effects. Interestingly, the pattern of intracellular mechanisms seems to be different for various class members.

Activation of AMPK, for example, has only been shown for canagliflozin but not for dapagliflozin or empagliflozin [86]. However, a pathway most likely influenced by all SGLT-2 inhibitors in cardiomyocytes is the Na^+/H^+ exchanger 1 (NHE1) mediated decrease in intracellular Na^+ and Ca^{2+}, although this has only been reported for empagliflozin so far. Decreased intracellular Ca^{2+} is likely to result in a negative inotropic effect; however, this is not necessarily the case if both systolic and diastolic Ca^{2+} decrease and the Ca^{2+} transient remains stable. Moreover, Baartscheer et al. did show that mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) did significantly increase upon empagliflozin administration. $[Ca^{2+}]_m$ signaling is critical for energy production as well as the activation of cell death pathways which are implicated in the development of heart failure [87]. These three changes in intracellular ion homeostasis counteract the alterations typically seen in heart failure models (e.g., elevated levels of intracellular Na^+ and Ca^{2+} and reduced levels of mitochondrial Ca^{2+} in heart failure) and might thus explain at least in part beneficial effects as seen in the EMPA-REG-OUTCOME trial [81].

Elevated diastolic Ca^{2+} also results in impaired relaxation and therefore diastolic dysfunction. Interestingly, empagliflozin significantly improved diastolic function in a rodent model of diabetes and reduced the expression of profibrotic and prohypertrophic proteins [88]. These effects could not be explained by reduced blood pressure levels as reported in several other models after SGLT-2 treatment, indicating towards a direct myocardial effect.

This idea is also supported by the finding that dapagliflozin but not pioglitazone significantly improves cardiac

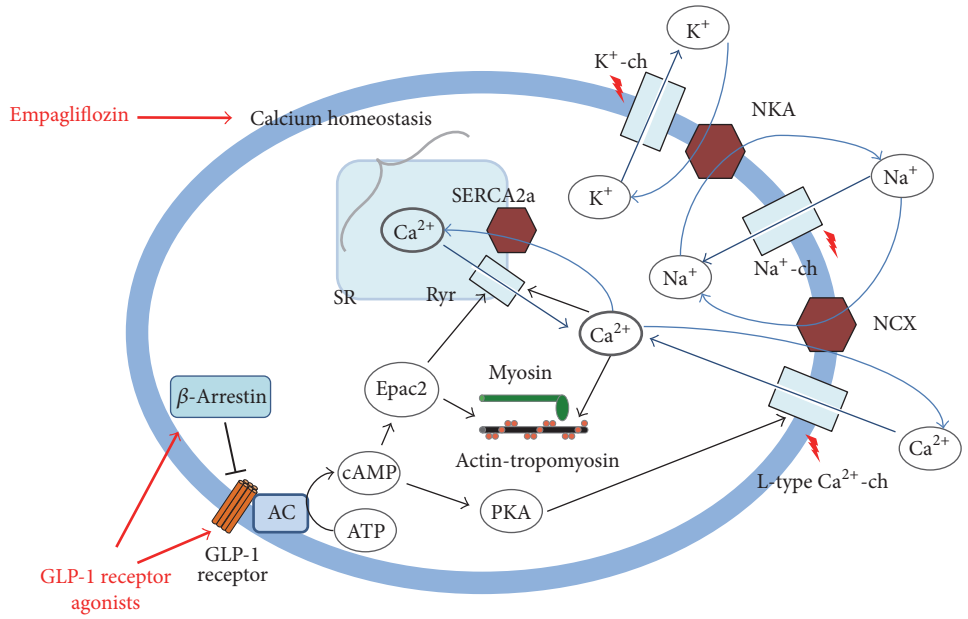


FIGURE 2: Interactions of antidiabetic drugs with cardiomyocytes: the well-established downstream mechanism of GLP-1 receptor agonists alters intracellular Ca^{2+} transients via a PKA-dependent activation of L-type Ca^{2+} channels and Epac2-dependent activation of the ryanodine receptor. For empagliflozin, potential downstream mechanisms are still unknown, yet there is strong evidence that the Ca^{2+} homeostasis is influenced. Possible downstream mechanisms of DPP-4 inhibitors are also still unknown. The wide interactions with cardiomyocytes via miscellaneous second messengers are not shown. (Na^+-ch: voltage gated sodium channel, K^+-ch: voltage gated potassium channel, Ryr; ryanodine receptor, NCX: sodium-calcium exchange pump, and NKA: sodium-potassium exchange pump).

function in a mouse model despite comparable glucose lowering effects. Ejection fraction and isovolumetric relaxation time were not altered in pioglitazone, but E/A ratio and ventricular hypertrophy were both slightly improved.

6. Metformin

In the United Kingdom Prospective Diabetes Study (UKPDS), 342 patients with an ideal body weight greater than 120% were randomly assigned to an intensive treatment with metformin or conventional treatment. A 39% relative risk reduction in fatal and nonfatal myocardial infarction ($p = 0.010$) and a 36% relative risk reduction in all-cause mortality ($p = 0.011$) were recorded in this study arm [89]. This finding in a limited number of patients is supported by data from a meta-analysis performed with randomized clinical trials data [90] suggesting a cardiovascular benefit associated with metformin. A larger trial investigating the effect of metformin in nondiabetic hyperglycemia is currently ongoing (ISRCTN 34875079).

7. Conclusion

With new and emerging primarily antihyperglycemic drugs, the intersection of antidiabetic treatment and cardiovascular therapy is progressing. Besides modulating diabetes as a cardiovascular risk factor several new antidiabetic drugs imply direct cardiovascular effects and in some cases these effects seem to directly affect myocardial tissue (Figure 2). Cardiovascular outcome trials requested by the FDA and EMA were designed to test for global and rather indirect cardiovascular effects and the mechanistic basis for the beneficial findings in some of these trials remain to be elucidated. Although these trials are called placebo-controlled trials, subjects in the control arm receive usual diabetes care excluding compound/drug class used in the active arm and all these trials aim for glycemic equipoise in both groups in order to exclude cardiovascular effects which could be due to differences in glycemic control [91].

Global assessment of cardiovascular outcome in usually short- to mid-term trials in particular high risk populations, however, might miss distinct intracellular effects of new antidiabetic drugs that could mediate more specific, positive or negative, effects on excitation-contraction coupling, contractility, metabolism, or energetics resulting in altered structural or functional properties.

An in-depth examination of cardiovascular outcome data in conjunction with basic science data is critical for a detailed understanding of benefits and risks of new antihyperglycemic drugs.

Abbreviations

DPP-4: Dipeptidyl peptidase 4
EMA: European Medicines Agency
eNOS: Endothelial NO-synthase
FDA: Food and Drug Administration
GLP-1: Glucagon-like peptide 1
GLUT: Glucose transporter
HFpEF: Heart failure with preserved ejection fraction

MACE: Major adverse cardiac event
NO: Nitric oxide
SERCA2a: Sarco-/endoplasmic reticulum Ca^{2+}-ATPase
SGLT: Sodium-dependent glucose transporter.

Conflicts of Interest

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

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Research Article

Mortality and Cardiovascular Complications in Older Complex Chronic Patients with Type 2 Diabetes

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Aims/Introduction. Determining the prevalence of diabetes and its cardiovascular complications and all-cause mortality in older chronic complex patients. *Materials and Methods*. We carried out a multicenter retrospective study and included a randomized sample of 932 CCP people. We assessed the prevalence of diabetes according to World Health Organization criteria. Data included demographics and functional, comorbidity, cognitive, and social assessment. *Results*. The prevalence of diabetes was 53% and average age 81.16 ± 8.93 years. There were no significant differences in the survival of CCP patients with or without DM, with or without ischaemic cardiopathy, and with or without peripheral vascular disease. The prognostic factors of all-cause mortality in patients with DM were age ≥ 80 years [HR 1.47, 95% CI 1.02–2.13, p 0.038], presence of heart failure [HR 1.73, 95% CI 1.25–2.38, p 0.001], Charlson score [HR 1.20, 95% CI 1.06–1.36, p 0.003], presence of cognitive impairment [HR 1.73, 95% CI 1.24–2.40, p 0.001], and no treatment with statins [HR 1.49, 95% CI 1.08–2.04, p 0.038]. *Conclusions*. We found high prevalence of DM among CCP patients and the relative importance of traditional risk factors seemed to wane with advancing age. Recommendations may include relaxing treatment goals, providing family/patient education, and enhanced communication strategies.

1. Introduction

The World Health Organization [1] has reported an increase in the ageing population living with major chronic diseases such as diabetes, dementia, cardiovascular disease, and certain cancers, with most of the increase in developing countries. Health experts have called this phenomenon the "grey tsunami" [2] due to its impact on the health system. Type-2 diabetes is one of the most common chronic diseases affecting older people and its prevalence increases with age. It has been estimated that the number of people over 65 with diabetes will increase by 4.5-fold by 2050 [3]; and diabetes is linked to higher mortality, reduced functional status, impaired quality of life, increased risk of institutionalization [4], and mortality.

Several publications have described the spectrum of comorbidities and functional impairment in ageing populations [5–7]. They emphasize a number of key features such as the emergence of cognitive dysfunction and frailty that can worsen adverse outcomes of diabetes such as emergency visits, increased fall risk, and mortality. The main goal of this study was to determine the prevalence of diabetes and its cardiovascular complications and all-cause mortality in complex chronic patients.

2. Materials and Methods

2.1. Study Population and Data Collection. This cohort study (2013–2016) included 3,490 cases registered as *complex chronic patient* enrolled in a large, integrated health primary care teams in the *Terres de l'Ebre* health area in Catalonia (Spain) with a sampling frame that included a randomized sample of 932 members. The source population was identified from the Catalonian Health Institute Registry as of 1 January 2013 to 31 December 2014. We included subjects if they met at least four of the following *criteria*: (1) age (≥65 years old), (2) chronic comorbidities (≥4), (3) psychosocial disorders (cognitive impairment or psychological disorder with functional disability), (4) geriatric conditions such as functional disability (Barthel score < 55, living in assisted living, in nursing home, or with in-home caregivers) or recurrent falls or fall risk, (5) previous high healthcare use (two hospitalizations not programmed for exacerbation of chronic pathologies or three emergency department visits in the last year), (6) ≥4 active medications in the last 6 months, and (7) living alone or with a caregiver ≥ 75 years old. Established in January 2013, the registry is written, managed, and updated by the nursing service in primary care using the *Shared Individual Intervention Plan* [*pla d'intervenció individualitzat compartit* (*PIIC*)]. Follow-up of this cohort members was initiated on 1 January 2013, and individuals were censored at the first occurrence of death that had occurred from any cause or at the end of the study (30 September 2016).

2.2. Variables. We collected data on demographic characteristics and data related to clinical, functional, cognitive, and social assessment. Comorbid conditions are defined using standard outpatient and inpatient ICD-9 codes by electronic data capture including pharmacy records, laboratory data, and outpatient, emergency room, and hospitalization diagnoses across all primary care centers and hospital. Charlson comorbidity index, short version, was scored. Polypharmacy was defined as five or more daily medications. If there was a diagnosis of atrial fibrillation (AF), $CHA_2DS_2VAS_C$HAS-BLED scores were included. Presence of cognitive impairment, a disease-specific diagnosis of cognitive impairment, without specification of subtype or severity, was measured using the Pfeiffer test. The variable definition includes recurrent falls or fall risk and presence of disability by Barthel score to assess dependence in ADL.

Currently, 82% of people registered as CCP have available clinical data in their PIIC report.

2.3. Criteria for the Definition of Diabetes. Diabetes (DM) was diagnosed according to any of the following WHO criteria: fasting plasma glucose (FPG) ≥ 7.0 mmol/l (126 mg/dl) or 75 g oral glucose tolerance test (OGTT) with FPG ≥ 7.0 mmol/l (126 mg/dl) and/or 2-hour plasma glucose ≥ 11.1 mmol/l (200 mg/dl) or HbA1c ≥ 6.5%/48 mmol/mol or random plasma glucose ≥ 11.1 mmol/l (200 mg/dl) in the presence of classical diabetes symptoms.

2.4. Statistical Analysis. Time to event analysis was performed using the Kaplan-Meier and Log Rank test. To estimate hazard ratios, mean survival time, and survival probabilities, we used a multivariate Cox regression. Multivariate Cox proportional hazards regression models were fitted to identify significant variables associated with the time to death since diagnosis as CCP. The adjusted model included the following baseline characteristics and the differences observed between DM and no DM and predictive factors for each event: age, sex, Charlson index, and factors in $CHA_2DS_2VAS_C$ scales and active pharmacy. The analyses were performed using IBM SPSS version 19.0.

3. Results

The baseline characteristics of the CCP group are shown in Table 1. The prevalence of CCP was 1.94% in the total population and 7.01% in those ≥60 years old. Diabetes had been diagnosed in 53% of the CCP population, with an average age of 81.16 ± 8.93 years, significantly younger ($p <$ 0.001) than those CCP without DM, but with a higher cardiovascular risk (p 0.015), a higher risk of stroke ($p <$ 0.001), more chronic conditions ($p < 0.001$), and a higher number of prescribed drugs ($p < 0.001$), but a higher Barthel score (p 0.003) and lower prevalence of cognitive impairment (p 0.001).

The patients were divided into three major subgroups defined jointly by age (<70, 70–79, and ≥80 years) and prevalence of cardiovascular complications (Table 2). There was a steady increase in the prevalence of cardiovascular comorbidities such as atrial fibrillation and heart failure, cognitive impairment, loss of autonomy in basic daily activities, increased fall risk, and the all-cause mortality rates associated with ageing. Other traditional risk factors associated with diabetes such as hypertension, dyslipidemia, and macrovascular complications such as ischaemic cardiopathy and peripheral artery disease stayed the same or even decreased with ageing.

The average follow-up time was 2.75 years (95% CI 2.42–3.07). The all-cause mortality rate was 32.8% in DM and 35.8% in non-DM patients. The incidence rate of death was 13.1/100 person-years in DM and 11.7/100 person-years in non-DM patients. There were no significant survival differences (Figure 1) between those with or without DM, with or without ischaemic cardiopathy (Figure 2), or with or without peripheral artery disease. There was a significant survival difference in the case of atrial fibrillation (p 0.003) (Figure 3) and heart failure ($p < 0.001$) (Figure 4). Table 3 shows number of deaths by decade of age.

In the CCP population with DM the prognostic mortality factors identified by the multivariate method were

Table 1: Baseline characteristics of CCP with and without diabetes.

CCP patients	No diabetes	Diabetes	p
N (%)	438 (47.00%)	494 (53.00%)	
Age (average ± SD)	84.22 ± 10.6	81.16 ± 8.0	<0.001
Percentage >80 years old n (%)	340 (77.6%)	315 (63.8%)	<0.001
Women n (%)	236 (53.9%)	252 (51.0%)	0.394
CCP criteria number (average ± SD)	3.85 ± 1.26	3.86 ± 1.12	0.955
Hypertension n (%)	362 (80.4%)	422 (85.4%)	0.044
Dyslipidemia n (%)	195 (44.5%)	326 (66.0%)	<0.001
Atrial fibrillation n (%)	176 (40.2%)	149 (30.2%)	0.002
Ischaemic cardiopathy n (%)	87 (19.9%)	109 (22.1%)	0.422
Peripheral artery disease n (%)	58 (13.2%)	91 (18.4%)	0.032
Chronic kidney insufficiency < 30 mlClCr	43 (9.8%)	73 (14.7%)	0.042
Heart failure n (%)	153 (34.9%)	150 (30.4%)	0.142
Charlson score (average ± SD)	2.05 ± 1.30	2.95 ± 1.30	0.015
Stroke before CCP n (%)	104 (23.7%)	96 (19.4%)	0.111
Stroke after CCP n (%)	36 (8.2%)	30 (6.1%)	0.249
$CHA_2DS_2VAS_C$ score (average ± SD)	5.97 ± 2.38	7.14 ± 5.97	<0.001
HAS_BLED score (average ± SD)	2.88 ± 1.14	3.10 ± 1.05	0.077
Daily medications number (average ± SD)	7.8 ± 3.29	9.80 ± 3.60	<0.001
Polypharmacy ≥ 4 n (%)	392 (89.5%)	484 (98%)	<0.001
Polypharmacy ≥ 10 n (%)	142 (32.4%)	258 (52.2%)	<0.001
Cognitive impairment n (%)	179 (40.9%)	159 (32.2%)	0.006
Pfeiffer test score (average ± SD)	3.43 ± 3.39	2.72 ± 3.13	0.001
Barthel score (average ± SD)	62.8 ± 32.41	69.02 ± 31.28	0.003
Barthel score < 60 n (%)	182 (41.6%)	161 (32.6%)	0.005
Fall risk n (%)	103 (23.9%)	85 (17.2%)	0.018
Gijón score (average ± SD)	9.53 ± 5.12	10.46 ± 3.87	0.363
Antiaggregant treatment n (%)	147 (33.6%)	238 (48.2%)	<0.001
Anticoagulant treatment n (%)	147 (33.6%)	123 (24.9%)	0.003
Statin treatment n (%)	149 (34.0%)	274 (55.5%)	<0.001
Proton pump inhibitor treatment n (%)	285 (65.1%)	358 (72.5%)	0.016
Selective serotonin reuptake inhibitors (SSRIs) n (%)	131 (29.9%)	149 (30.2%)	0.943
CNS depressant drugs n (%)	249 (56.8%)	265 (53.6%)	0.356
Death n (%)	157 (35.8%)	162 (32.8%)	0.334

age ≥ 80 years [HR 1.47, 95% CI 1.02–2.13, "p 0.038"], heart failure [HR 1.73, 95% CI 1.25–2.38, "p 0.001"], the Charlson score [HR 1.20, 95% CI 1.06–1.36, "p 0.003"], cognitive impairment [HR 1.73, 95% CI 1.24–2.40, "p 0.001"], and no treatment with statins [HR 1.49, 95% CI 1.08–2.04, "p 0.038"].

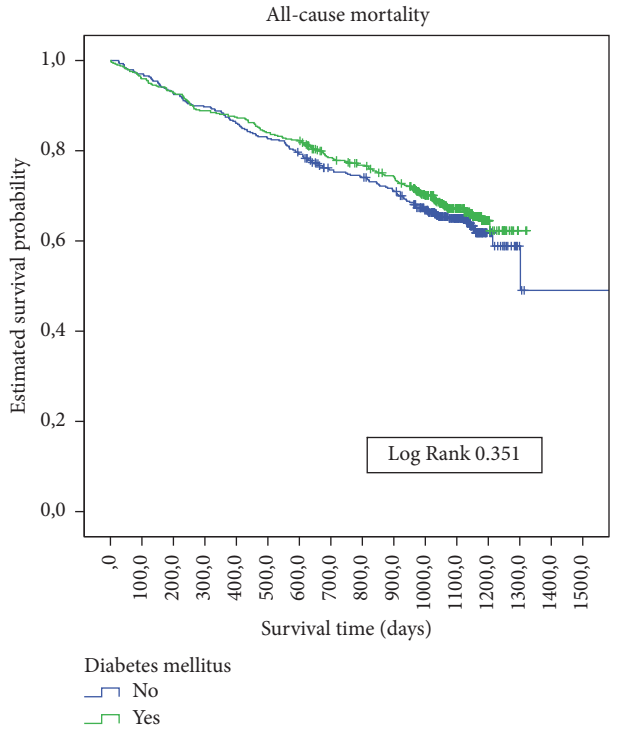

Figure 1: Kaplan-Meier estimates of survival during follow-up in CCP with or without diabetes mellitus at baseline.

We can define an epidemiological model with a high prevalence of DM (53%) associated with classical risk factors such as hypertension (84.1%), dyslipidemia (52.1%), atrial fibrillation (37.9%), and stroke (28.3%) to which other risk factors can be added such as age ≥80 years (70.2%), cognitive impairment (44.1%), heart failure (35.6%), CNS depressant drugs (56.9%), fall risk (23.2%), and the lowest Barthel score (59, 95% CI 57.1–62.08), all of which increase the risk of mortality.

4. Discussion

The registered prevalence of CCP in our study was higher than in other developed countries [8] as was the prevalence of diabetes mellitus in our CCP population [2, 9, 10]. The frequency of comorbidity burden such as cognitive impairment, polypharmacy, functional disability of basic activities of daily living, and limited availability of caregiver support may be a substantial problem in implementing a management plan. A consensus has developed on how to treat older people with diabetes [11, 12], but given that complications may not be present or may take many years to develop, efforts should be adapted depending on the average remaining life expectancy and degree of impairment of quality of life.

The authors believe a strategic change will involve moving away from "*the diabetic patient with complications*" to "*CCP with chronic comorbidities.*" Therefore, while classically diabetes mellitus has been associated with the development

Table 2: Baseline characteristics of CCP patients according to age.

CCP patients	<70 years old	70–79 years old	≥80 years old
N (% all)	93 (9.97%)	184	655
Age (average CI 95%)	61.39 (59.6–63.1)	75.6 (75.2–76.02)	87.5 (87.2–87.9)
Women *n* (% group)	41/93 = 44.08%	82 (44.6%)	365 (55.7%)
CCP criteria number (average CI 95%)	2.9 (2.7–3.22)	3.69 (3.5–3.8)	4.02 (3.94–4.12)
Diabetes mellitus *n* (% group)	53 (57.%)	125 (67.9%)	16 (48.2%)
Hypertension *n* (% group)	63 (67.7%)	160 (87.0%)	551 (84.1%)
Dyslipidemia *n* (% group)	55 (59.1%)	125 (67.9%)	341 (52.1%)
Atrial fibrillation *n* (% group)	17 (18.3%)	60 (32.6%)	248 (37.9%)
Ischaemic cardiopathy *n* (% group)	20 (21.5%)	43 (23.4%)	133 (20.3%)
Peripheral artery disease *n* (% group)	17 (18.3%)	41 (22.3%)	91 (13.9%)
Chronic kidney insufficiency < 30 mlClCr *n* (% group)	12 (12.9%)	24 (13.1%)	80 (12.2%)
Heart failure *n* (% group)	17/93 = 18.27%	53 (28.8%)	233 (35.6%)
Charlson score (average CI 95%)	2.17 (1.87–2.47)	2.7 (2.5–2.9)	2.52 (2.42–2.63)
Stroke *n* (% group)	21 (22.6%)	51 (27.8%)	185 (28.3%)
$CHA_2DS_2VAS_C$ score (average CI 95%)	4 (3.11–4.9)	5.17 (4.84–5.51)	5.03 (4.88–5.20)
Stroke risk/year (average CI 95%)	4.78 (3.55–6.02)	6.81 (6.2–7.4)	6.57 (6.27–6.87)
Daily medications number (average CI 95%)	9.21 (8.37–10.6)	10.1 (9.5–10.53)	8.5 (8.24–8.77)
Cognitive impairment *n* (% group)	14 (15.1%)	35 (19.0%)	289 (44.1%)
Pfeiffer test score (average CI 95%)	1.40 (0.86–1.95)	1.53 (1.19–1.89)	3.71 (3.46–3.97)
Barthel score (average CI 95%)	82.8 (77.3–88.4)	80.67 (76.9–84.4)	59. (57.1–62.08)
Fall risk *n* (% group)	5 (5.4%)	31 (16.8%)	152 (23.2%)
Antiaggregant treatment *n* (% group)	30 (32.3%)	82 (44.6%)	273 (41.7%)
Anticoagulant treatment *n* (% group)	25 (26.9%)	55 (29.9%)	175 (26.7%)
HAS_BLED (average CI 95%)	2.93 (2.22–3.65)	3.06 (2.81–3.31)	2.96 (2.82–3.11)
Bleeding risk/year (average CI 95%)	5.18 (3.0–7.37)	4.89 (4.0–5.8)	4.95 (4.5–5.41)
Statin treatment *n* (% group)	54 (58.1%)	118 (64.1%)	251 (38.3%)
CNS depressant drugs *n* (% group)	44 (47.3%)	97 (52.7%)	373 (56.9%)
Death *n* (% group)	12 (12.9%)	48 (26.1%)	259 (39.5%)
Average follow-up time *n* (days CI 95%)	1348 (719–1977)	947 (901–993)	971 (825–1117)

of micro and macrovascular complications, with an ageing population, it is not yet clear whether the presence of chronic cardiovascular comorbidities or a higher risk of undergoing acute cardiovascular events (higher $CHA_2DS_2VAS_C$ score) should be approached as preventable and modifiable risk factors (antiaggregant, statins) or as one more condition of the "chronic complex patient" that needs to be controlled but without benefits for cardiovascular risk.

Given that it is unknown how long the DM is running and that most risk score tables exclude 75% of our CCP due to their age, the use of such tables is pointless because we cannot relate the cardiovascular complication rates to a risk score. On the other hand, strategies to modify the incremental tendency for DM involving the promotion of healthy lifestyles (mainly associated with diet and physical activities) and the detection of high risk patients are difficult to apply at this vital stage. The priorities should be avoiding or lowering the risk of complications (hypoglycemia, hyperglycemia, fall, and polypharmacy) via an appropriate stratification using the functional autonomy based on adaptations to individual environments. The literature related to interventions for lifestyle diseases in developing countries is very limited, probably due to the multitude of possible health endpoints and interventions, the multiple sources of the problem, and the limited knowledge of means of changing individual and population behavior [13–15].

In contrast with other studies [16], our results show that the incidence of all-cause mortality and the prevalence of cardiovascular diseases do not differ significantly among CCP with and without DM. The data about deaths related to diabetes are confusing and come from different age cohorts: according to the World Health Organization, 43% of all deaths due to high blood glucose occur before the age of 70 [17]; and the proportion of deaths attributable to diabetes was estimated to be 11.5% in the United States [18, 19]. In our study the mortality rate due to diabetes was 17%, and <70 years old is when the difference in mortality is higher between DM and no DM. This fact supports our thought of a different epidemiologic profile in this group of population. However, we found risk factors associated with ageing such as cognitive impairment and heart failure that have been described previously as prognostic factors for mortality [5–7] in the CCP population. This supports the idea that all-cause mortality is more affected by ageing factors than by specific

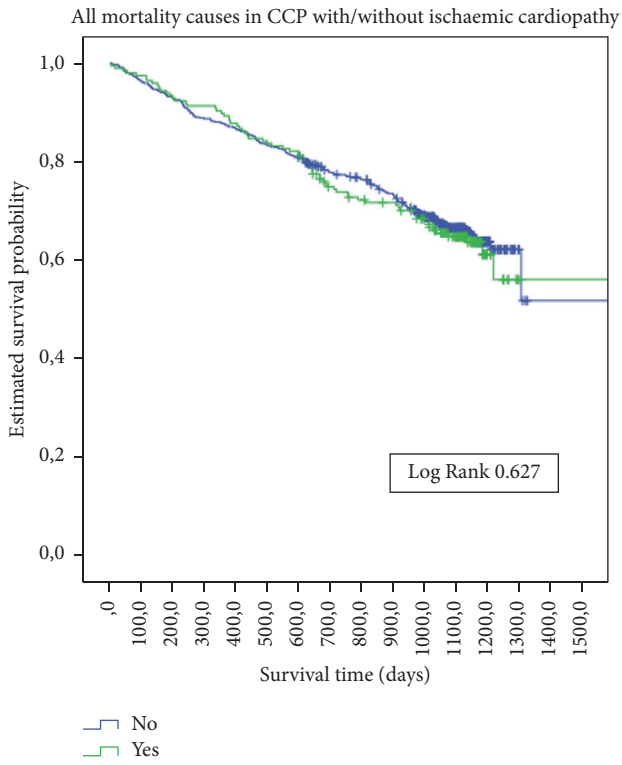

Figure 2: Kaplan-Meier estimates of survival during follow-up in CCP with or without ischaemic cardiopathy at baseline.

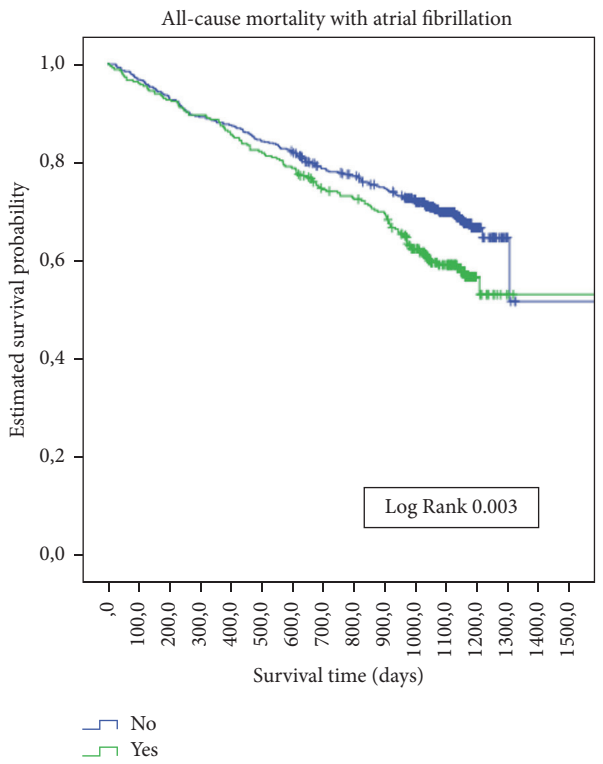

Figure 3: Kaplan-Meier estimates of survival during follow-up in CCP with or without atrial fibrillation at baseline.

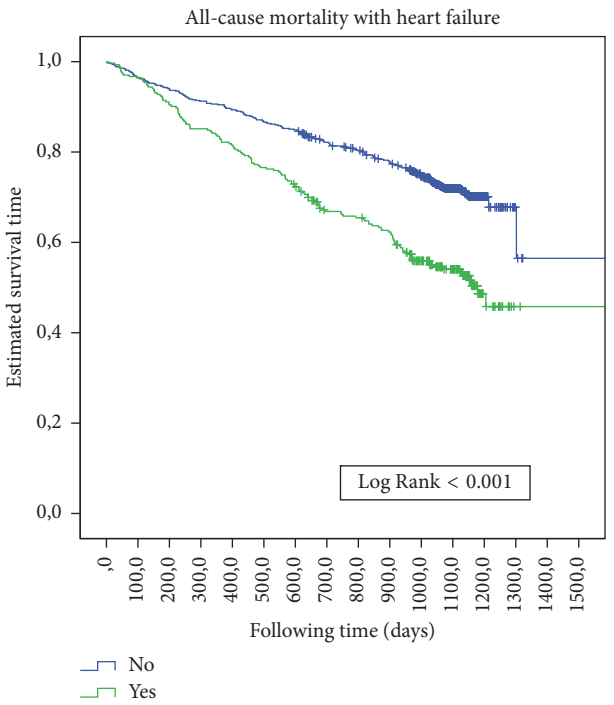

Figure 4: Kaplan-Meier estimates of survival during follow-up in CCP with and without heart failure at baseline.

Table 3: Number of deaths according to age.

Age	Diabetes *N* (%)	No diabetes *N* (%)	*p*	All
<70 (*n* 93)	9 (17.0%)	3 (7.5%)	0.150	12 (12.9%)
70–79 *n* (184)	32 (25.6%)	16 (27.1%)	0.480	48 (26.1%)
80–89 *n* (435)	74 (31.6%)	72 (35.8%)	0.361	146 (33.6%)
≥90 *n* (220)	47 (57.3%)	66 (47.8%)	0.209	113 (51.4%)
Total (*n* 932)	162 (32.8%)	157 (35.8%)	0.181	319 (34.2%)

complications of DM. Likewise the incidence of DM in this CCP population may be more of a consequence of the ageing process than an independent disease. The comorbidities may drive all-cause mortality and the contribution of diabetes in the presence of complex chronic diseases to overall mortality seems to be only minimal.

Despite the above, we should not underestimate the importance of evaluating and treating cardiovascular risk given that it plays a key role in cardiovascular prevention in all national and international guidelines [20], mainly among patients with a high level of functional dependence. The relative importance of traditional risk factors seems to wane with advancing age [21] and for this reason; we emphasize the importance of redefining the care strategy and adapting it to comorbidities and functional autonomy rather than

achieving excellent levels of glycated haemoglobin or total cholesterol.

The limitations of the present approach are related to the possibility of overestimating the prevalence of DM among the CCP population as a consequence of the definition criteria despite being a randomized sample; not knowing the duration of DM in order to prevent complications; and perhaps the frequency with which diabetes is listed as the underlying cause of death is not a reliable indicator of its contribution to the mortality profile. Also, we need to determine accurately whether diabetes was the underlying cause of death or was only an associated cause of death.

Conflicts of Interest

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

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Research Article

MMP-2, MMP-9, and TIMP-4 and Response to Aspirin in Diabetic and Nondiabetic Patients with Stable Coronary Artery Disease: A Pilot Study

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Background. High on-aspirin treatment platelets reactivity (HPR) is a significant problem in long-term secondary prevention of cardiovascular events. We hypothesize that imbalance between platelets MMPs/TIMPs results in cardiovascular disorders. We also explored whether chronically elevated blood glucose affects MMP-2/TIMP-4 release from platelets. *Materials and Methods*. Seventy patients with stable coronary artery disease, supplemented with aspirin, participated in this pilot study. The presence of HPR and/or diabetes mellitus was considered as the differentiating factor. Light aggregometry, impedance aggregometry, and ELISA tests for TXB2, MMP-2, MMP-9, and TIMP-4 were performed in serum, plasma, platelet-rich plasma, and platelets-poor plasma, as appropriate. *Results*. Aspirin-HPR did not affect plasma MMP-2, MMP-9, and TIMP-4. Arachidonic acid-induced aggregation of platelets from aspirin-HPR patients did not lead to increased release of MMP-2, MMP-9, and TIMP-4. Studying patients at the lowest TXB2 serum concentration quartile revealed that high concentration of plasma TIMP-4 and TIMP-4 negatively correlated with TXB2 and platelet aggregation. Diabetics showed an increased plasma MMP-2 as well as an increased MMP-2 in supernatants after platelet aggregation. However, diabetes mellitus did not affect MMP-9 and TIMP-4. *Conclusion*. Aspirin-HPR did not affect the translocation and release of MMPs and TIMP-4 from platelets. TIMP-4 may serve as a marker of TXA2-mediated platelet aggregation. Chronically elevated plasma glucose increases plasma MMP-2, and HPR potentiates this phenomenon.

1. Introduction

Diabetes mellitus (DM) is one of the major risk factors for the development of cardiovascular disease and a higher mortality [1]. It has been reported that patients with DM type 2 and no previous history of coronary artery disease (CAD) have similar risk for cardiac events to patients with prior myocardial infarction [2]. Apart from traditional risk factors for the development of cardiovascular events in diabetes subjects, nowadays a lot of attention is payed to nontraditional risk factors including haematological and thrombogenic factors. Atherothrombosis, defined as the formation of a thrombus on atherosclerotic plaque, is the leading cause of acute cardiovascular events [3]. Going further, it is well documented that hyperglycemia increases the expression and activity of matrix metalloproteinases (MMPs) in vascular macrophages and endothelial cells; hence it facilitates vascular remodeling and cardiovascular complications [4].

Matrix metalloproteinases are ubiquitous in the family of calcium-dependent zinc-containing endopeptidases that are mainly involved in the degradation and remodeling of extracellular matrix of the tissues. They are expressed at low

level in normal adult tissue turnovers such as reproduction [5, 6], development [7], tissue repair [8], or immune response [9, 10] and are upregulated during pathological processes including inflammation [11], autoimmune diseases [12], neurogenerative disorders [13], tumor invasion and metastasis [14, 15], and heart injury [16]. Broad substrate specificities and strict regulation of their expression, activation, and inhibition levels contribute to maintenance of tissue homeostasis. The activity of MMPs is regulated mostly by the endogenous tissue inhibitors of metalloproteinases (TIMPs), which bind to the active site of MMPs and block access to extracellular matrix substrates [17, 18]. Besides the extracellular role of MMPs, several studies describe an intracellular action of MMPs in physiological and pathological states [19–21] in which both MMP-2 and MMP-9 as well as TIMP-4 have been identified in platelets [22]. During aggregation, MMP-2 and MMP-9 are translocated from the cytosol to the platelet surface [22, 23] where MMP-2 remains in close association with platelet membrane adhesion receptors affecting their activation and the aggregatory response of platelets [24]; MMP-9 shows an opposite antiaggregatory activity [23, 25]. It was also reported that TIMP-4 is colocalized with MMP-2 in resting platelets and is released from platelets upon aggregation [26]. On the basis of this evidence we hypothesize that the dissociation of TIMP-4 from TIMP-4-MMP-2 complex and release of this proteins into extracellular space may regulate platelets aggregation.

Aspirin (acetylsalicylic acid) inhibits platelet aggregation by irreversible inactivation of cyclooxygenase enzyme (COX-1), which is involved in prostaglandins and thromboxane A_2 synthesis [27, 28]. As a factor of decreased risk of cardiovascular incidents, it is widely used in clinical practice during coronary interventions and in long-term secondary prevention of cardiovascular and cerebrovascular events. However, in some patients, a high on-aspirin treatment platelet reactivity (HPR), referred to as a higher than expected platelet reactivity in patients under antiplatelet therapy, is observed. The limited degree of inhibition of platelet function is associated with poor cardiovascular outcomes and might be of clinical value for identifying patients with high risk of recurrent vascular events who may benefit from intensified antiplatelet therapy [29]. While HPR has been widely described in many papers, a precise mechanism has not been clearly explained. There are some contrary results showing an influence of aspirin on MMP-2/TIMP pathways in platelets. Falcinelli et al. (2007) and others showed that treatment with aspirin did not affect the translocation and release of MMP-2 from platelets [30, 31], but Hua et al. (2009) reported that aspirin decreased the expression and release of MMP-2 and MMP-9 from monocytes [32]. Others showed that MMP-9 can influence the action of aspirin through modification of the TXA_2 pathway [23] and that aspirin can influence MMP-2 and MMP-9 production in monocytes [32] and megakaryocytes [33, 34]. Based on these discrepancies, the main aim of the current pilot study was to explore if MMPs/TIMP-4 interactions in platelets or plasma are associated with the response to aspirin in patients with diabetes and stable coronary artery disease and whether chronically elevated blood glucose affects MMP-2/TIMP-4 release from platelets.

2. Material and Methods

2.1. Study Group and Clinical Material. Seventy patients with stable coronary artery disease participated in this study. Clinical characteristics of study participants are presented in Table 1. All participants were recruited by the Department and Clinic of Cardiology, Wroclaw Medical University in Wroclaw. Written informed consent was obtained for the collection of blood samples. The study was approved by the local Ethics Committee of the Medical University of Silesia. Study subjects were informed in detail about the purpose and the principles of this study. 30 ml of citrate anticoagulated whole blood (1 + 9, v : v) was collected for further analysis.

2.2. Criteria for Classification. Inclusion criteria for the study included stable coronary artery disease and aspirin use in a dose of 75 mg per day for at least 7 days preceding study inclusion. Exclusion criteria incorporated intake of antiplatelet drugs other than aspirin during two weeks before study inclusion, percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG) up to 3 months before study inclusion, current bleeding and anemia, and platelets count in whole blood below 150,000/mm^3 or above 450,000/mm^3.

2.3. Light Aggregometry (LTA). Light aggregometry was performed with the use of Chronolog "560 Ca" aggregometer (Havertown, USA). Briefly, sodium citrate anticoagulated whole blood (0.109 M) was centrifuged at 100g for 15 minutes without braking to obtain platelet reach plasma (PRP). Half of the PRP volume was centrifuged again (20 minutes at 2400g) to obtain platelet poor plasma (PPP) which served as a blank. The platelet count in PRP was adjusted to 300,000/mm^3. Platelets aggregation was measured after the addition of arachidonic acid as an agonist (Chronolog, Havertown, USA) with a final concentration of 0.5 mg/ml. Maximum platelet aggregation during a 5-minute interval was assessed. The range of values for LTA was 0–100%. Results were given in percentage of light transmittance. Every aggregation measurement was performed in duplicate with the mean subtraction. If 10% difference between measurements and the mean appeared, additional aggregation measurements were performed.

2.4. Impedance Aggregometry (IMA). Multiplate Aggregometer was used (Roche, France) for impedance in the whole blood aggregation measurement. Blood was collected into tubes containing hirudin (25 μg/ml) (Sarstedt, Germany). Arachidonic acid in final concentration of 0.5 mM (Roche, France) was used as an agonist. Results were given as areas under aggregation curves in arbitral units (AU). Every aggregation measurement was performed in duplicate, and if the difference between measurements was above 10%, another two measurements were performed.

2.5. TXB2 Concentration in Serum. Blood was collected in dry tubes and then heated at 37°C for 60 minutes. Serum samples were collected by centrifugation. The concentration of serum TXB2 was measured by ELISA test (R&D, USA).

Table 1: Clinical characteristics of the study population.

Clinical parameter	Number of patients (%)		Statistical significance
	CAD, diabetes group	CAD, no-diabetes group	
Total number of patients	35 (50)	35 (50)	
Age (years), mean ± SEM	62.7 ± 1.5	60.1 ± 1.6	NS
Sex			
Men	27 (77.1)	28 (80.0)	NS
Women	8 (22.9)	7 (20.0)	NS
Clinical characteristics			
Arterial hypertension	29 (82.8)	28 (80.0)	NS
Current tobacco use	10 (28.5)	15 (42.8)	NS
History of myocardial infarction	21 (60.0)	13 (37.1)	NS
History of PCI/CABG	18 (51.4)/7 (20.0)	10 (28.6)/3 (8.6)	NS
History of stroke or TIA	3 (8.5)	2 (5.7)	NS
Kidney insufficiency (GFR < 60 ml/min/m^2),	10 (28.5)	5 (14.2)	NS
Hypercholesterolemia	35 (100)	30 (85.7)	NS
HbA1C, mean ± SD	6.8 ± 3.0	NA	NA
Drug administration			
Beta-blocker	35 (100)	32 (91.4)	NS
Calcium channel blockers	20 (57.1)	22 (62.8)	NS
ACE-I	30 (85.7)	31 (88.5)	NS
ARB	5 (14.2)	10 (28.5)	NS
Statins	35 (100)	35 (100)	NS
Oral antidiabetic drugs	30 (85)	NA	NA
Insulin	21 (65)	NA	NA
HPR criteria			
LTA (Amax > 20%)*	2 (5.7)	1 (2.8)	NS
LTA (Amax > 15%) (highest quartile)*	8 (22.8)	4 (11.4)	NS
MEA (AspiTEST > 30 AU)	10 (28.6)	9 (25.7)	NS
TXB2 > 3.1 ng/ml	15 (42.9)	11 (31.4)	NS
TXB2 > 5.8 ng/ml (highest quartile)	9 (25.7)	8 (22.9)	NS

Notes. ACE-I: angiotensin converting enzyme inhibitor; ARB: angiotensin receptor blocker; CABG: coronary artery bypass grafting; GFR: glomerular filtration rate; HbA1C: glycated hemoglobin A1C; LTA: light aggregometry; MEA: multielectrode aggregometry; NA: not analyzed; NS: not statistically significant; SD: standard deviation; PCI: percutaneous coronary intervention; TXB2: thromboxane B2; TIA: transient ischemic attack; *a range of values for LTA was 0–100%.

2.6. MMP-2, MMP-9, and TIMP-4 in Plasma and Supernatant of PRP. Sodium citrate anticoagulated blood was collected on ice and centrifuged (1000 ×g, 20 min, 4°C) immediately after collection and separated plasma samples were used for the assessment of metalloproteinases and their inhibitor concentrations. Commercially available ELISA tests for MMP-2 (Total MMP-2 Quantikine ELISA), MMP-9 (Human MMP-9 Quantikine ELISA), and TIMP-4 (Human TIMP-4 Quantikine ELISA) (R&D, USA) were used. Total MMP-2 including active MMP-2, pro-MMP-2, and TIMP complexed matrix metalloproteinase 2 as well as active and proenzyme of MMP-9 concentrations were measured. The minimum detectable dose (MDD) was on average 0.033 ng/mL for MMP-2, less than 0.156 ng/mL for MMP-9, and on average 4.91 pg/mL for TIMP-4. Total MMP-2 Quantikine ELISA assay recognized recombinant MMP-2 and natural human, mouse, rat, porcine, and canine active MMP-2, pro-MMP-2, and TIMP complexed MMP-2. Human MMP-9 Quantikine ELISA test was able to measure natural and recombinant 92 kDa pro-MMP-9 and the 82 kDa active MMP-9. It did not measure the 65 kDa form of MMP-9. In turn, Human TIMP-4 Quantikine ELISA recognized natural and recombinant human TIMP-4.

To study platelets release of MMP-2, MMP-9, and TIMP-4 into extracellular space, their concentration was also measured in supernatants of PRP after platelet aggregation (see optical aggregometry above). Additionally, to study an influence of aspirin on MMPs and TIMP-4 release from platelets, the optical aggregometry with arachidonic acid after 5-minute incubation of PRP with aspirin 100 μg/ml (Laspal, Polfa, Poland) was performed. Supernatants after aggregation and PPP used as a blank for LTA (obtained by blood centrifugation at room temperature) were also used to measure MMPs/TIMP-4 concentrations.

2.7. High On-Aspirin Treatment Platelet Reactivity (HPR). Aspirin-high on-treatment platelets reactivity was defined to be present when in vitro platelet reactivity (assessed by the use

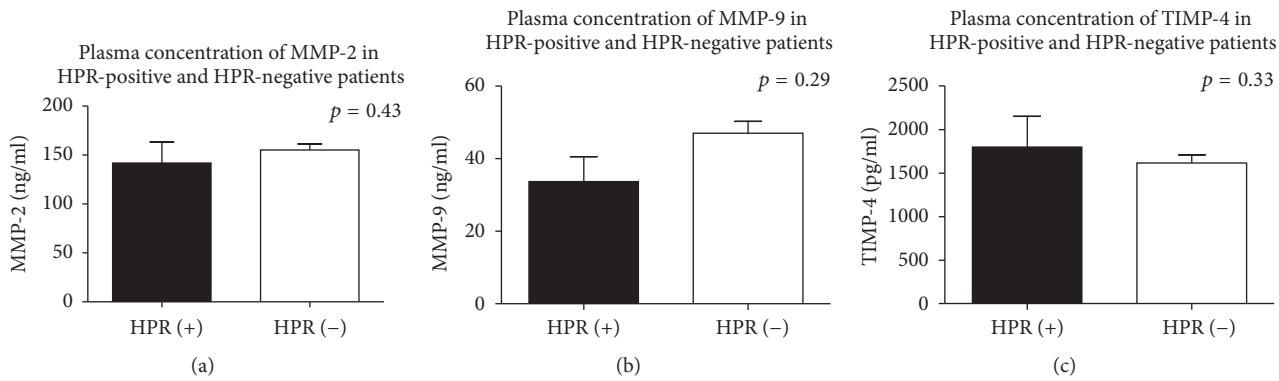

Figure 1: An influence of HPR on plasma concentration of MMP-2 (a), MMP-9 (b), and TIMP-4 (c). Mean ± SEM classification of HPR on the basis of LTA Amax > 20%. HPR-high on-aspirin treatment platelets reactivity; LTA: light transmittance aggregometry; MMP: matrix metalloproteinase; TIMP-4: tissue inhibitor of MMPs.

of single laboratory test) was not properly blocked despite the use of oral antiplatelet drugs, according to established criteria [28]. Five different criteria were used to determine HPR. HPR in light aggregometry (optical aggregation) was present if maximal aggregation exceeded 20% or in impedance method was above 30 AU (0 Ω). TXB2 level higher than 3.1 ng/ml was also considered to be HPR. Additionally, LTA and TXB2 level were divided into quartiles and correlated with MMPs/TIMP concentrations.

2.8. Statistics. Statistica 12 software (StatSoft, USA) was used for data analysis. Results were expressed as mean ± SEM or median (interquartile range). Data that showed a right-skewed distribution but met the remaining criteria for the normal distribution was transformed logarithmically and analyzed by relevant tests. Shapiro, one-way or two-way ANOVA and Mann-Whitney U tests were used as appropriate. To confirm the homogeneity of compared groups χ^2 test with Yates correction has been used. Spearman's or Kendall's rank correlation was used to assess the correlations.

3. Results

3.1. Plasma Concentration of MMP-2, MMP-9, and TIMP-4 in Patients with High On-Aspirin Treatment Platelets Reactivity (HPR). HPR was tested according to established criteria by use of light transmittance and multielectrode aggregometry or by measurement of serum TXB2. Data showed that plasma concentrations of MMP-2, MMP-9, and TIMP-4 were similar both in HPR patients and their counterparts (Figure 1). Since concordance among different tests in the identification of patients with HPR is limited [35], we have compared the concentrations of matrix metalloproteinases and their inhibitor in HPR patients classified by different methods. Despite testing different criteria for aspirin-HPR (cut off point >15%, >20%, >30 AU, >3.1 ng/ml, and >5.8 ng/ml, as appropriate), we did not show a significant difference in MMP-2, MMP-9, and TIMP-4 concentrations in plasma of aspirin responding and aspirin-HPR patients (Table 2).

3.2. MMP-2, MMP-9, and TIMP-4 Release from Aggregating Platelets Obtained from HPR-Positive and HPR-Negative Patients. To determine whether insufficient platelet inhibition in patients with high on-aspirin treatment platelets reactivity affects platelets MMPs/TIMP-4 pathway, the concentration of MMP-2, MMP-9, and TIMP-4 in supernatants obtained due to platelets aggregation was determined. We reported that arachidonic acid-induced aggregation of platelets from aspirin-HPR patients did not reveal an increased release of MMP-2, MMP-9, and TIMP-4 in comparison to patients with sufficient platelet inhibition (Table 2, Figures 2(a)–2(c)).

Additionally, to study a direct influence of aspirin on MMPs and TIMP-4 release from platelets, an optical aggregometry with arachidonic acid after 5-minute incubation of platelet-rich plasma with aspirin was performed. We showed that mean concentrations of MMP-2, MMP-9, and TIMP-4 in supernatants after aggregation of aspirin preincubated platelets were similar in patients with aspirin-HPR and in patients with properly inhibited platelets (Table 2, Figures 2(d)–2(f)).

We also measured the concentration of MMPs/TIMPs in PPP (used as a blank for LTA), obtained by blood centrifugation at room temperature. There was no significant difference ($p > 0.05$) in MMPs/TIMPs concentration between following samples: PPP from LTA obtained at room temperature versus plasma obtained from cold centrifugation versus PRP supernatants after aggregation versus PRP supernatants after aggregation with aspirin preincubation.

3.3. An Influence of TIMP-4 on Production of TXB2 and Platelets Aggregation. We found that patients at the lowest TXB2 serum concentration quartile (below 1.2 ng/ml) had higher antiaggregatory TIMP-4 plasma concentration in comparison to patients with higher TXB2 concentration (2195.8±942.3 versus. 1325.0±526.5; $p = 0.023$) (Figure 3(a)). Plasma TIMP-4 negatively correlated with TXB2 ($r = -0.24$, $p = 0.014$) (Figure 3(b)) and platelets aggregation ($r = -0.27$, $p = 0.039$) (Figure 3(c)).

Table 2: MMP-2, MMP-9, and TIMP-4 concentrations in aspirin good responders and aspirin-HPR patients (according to different criteria).

MMPs/TIMP-4	LTA (Amax > 20%)		p value	LTA (Amax > 15%) (highest quartile)		p value	MEA (AspiTEST > 30 AU)		p value	TXB2 > 3.1 ng/ml		p value	TXB2 > 5.8 ng/ml (highest quartile)		p value
	HPR + (n = 3)	HPR − (n = 67)		HPR + (n = 12)	HPR − (n = 58)		HPR + (n = 19)	HPR − (n = 51)		HPR + (n = 26)	HPR − (n = 44)		HPR + (n = 17)	HPR − (n = 53)	
Plasma level															
MMP-2 (ng/ml)	141.8 ± 21.2	155.1 ± 6.38	0.43	153.4 ± 11.3	154.8 ± 7.0	0.09	168.6 ± 15.1	149.9 ± 6.1	0.16	161.9 ± 12.4	149.7 ± 6.5	0.08	137.6 ± 7.9	160.6 ± 7.8	0.11
MMP-9 (ng/ml)	33.7 ± 6.8	47.0 ± 3.3	0.29	49.1 ± 5.4	45.9 ± 3.6	0.17	53.4 ± 6.6	43.6 ± 3.5	0.55	50.2 ± 4.8	41.4 ± 3.8	0.14	51.5 ± 7.0	42.6 ± 3.1	0.78
TIMP-4 (pg/ml)	1796.3 ± 354.1	1614.1 ± 94.4	0.33	1757.5 ± 222.8	1593.9 ± 99.7	0.44	1502.9 ± 136.8	1676.9 ± 114.1	0.17	1379.7 ± 106.7	1726.2 ± 127.8	0.87	1466.5 ± 140.0	1630.5 ± 112.2	0.06
Supernatant of PRP after LTA induced by AA															
MMP-2 (ng/ml)	141.1 ± 21.3	153.6 ± 6.0	0.70	153.3 ± 10.0	153.1 ± 6.7	0.06	166.4 ± 14.4	148.0 ± 5.9	0.90	157.1 ± 11.7	149.7 ± 6.3	0.15	134.2 ± 8.4	159.2 ± 7.3	0.13
MMP-9 (ng/ml)	33.0 ± 7.2	44.7 ± 2.67	0.12	48.3 ± 5.8	43.3 ± 2.8	0.34	43.3 ± 4.5	43.9 ± 3.1	0.14	43.9 ± 3.3	43.8 ± 3.5	0.78	42.8 ±.4.5	44.2 ± 3.0	0.62
TIMP-4 (pg/ml)	1762.6 ± 346.2	1568.9 ± 89.9	0.45	1694.0 ± 207.7	1553.0 ± 95.4	0.09	1465.3 ± 128.7	1629.6 ± 108.8	0.07	1364.5 ± 101.8	1662.4 ± 122.4	0.80	1440.9 ± 136.7	1579.4 ± 106.5	0.76
Supernatant after 5 minutes of aspirin incubation and subsequent LTA induced by AA															
MMP-2 (ng/ml)	135.6 ± 19.2	146.9 ± 5.9	0.22	148.1 ± 10.9	146.0 ± 6.5	0.08	157.7 ± 12.5	142.9 ± 6.2	0.08	151.6 ± 11.8	142.8 ± 6.4	0.08	129.2 ± 7.4	152.4 ± 7.2	0.34
MMP-9 (ng/ml)	35.8 ± 6.5	44.7 ± 2.8	0.53	51.7 ± 7.8	42.8 ± 2.8	0.41	40.6 ± 3.7	45.4 ± 3.4	0.36	45.1 ± 2.8	42.6 ± 3.5	0.78	44.2 ± 4.0	43.4 ± 3.0	0.08
TIMP-4 (pg/ml)	1748.0 ± 340.3	1548.4 ± 91.4	0.07	1669.4 ± 208.9	1533.7 ± 97.7	0.76	1459.3 ± 130.8	1604.1 ± 110.7	0.73	1360.7 ± 103.2	1632.7 ± 124.8	0.67	1439.7 ± 140.6	1553.7 ± 107.8	0.16

Notes. AA: arachidonic acid; AspiTEST: arachidonic acid-induced aggregation in MEA; LTA: light transmittance aggregometry; MEA: multielectrode aggregometry; MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9; TIMP-4: tissue inhibitor of matrix metalloproteinase-4; TXB2: thromboxane B2; HPR: high on treatment platelet reactivity; mean ± SEM.

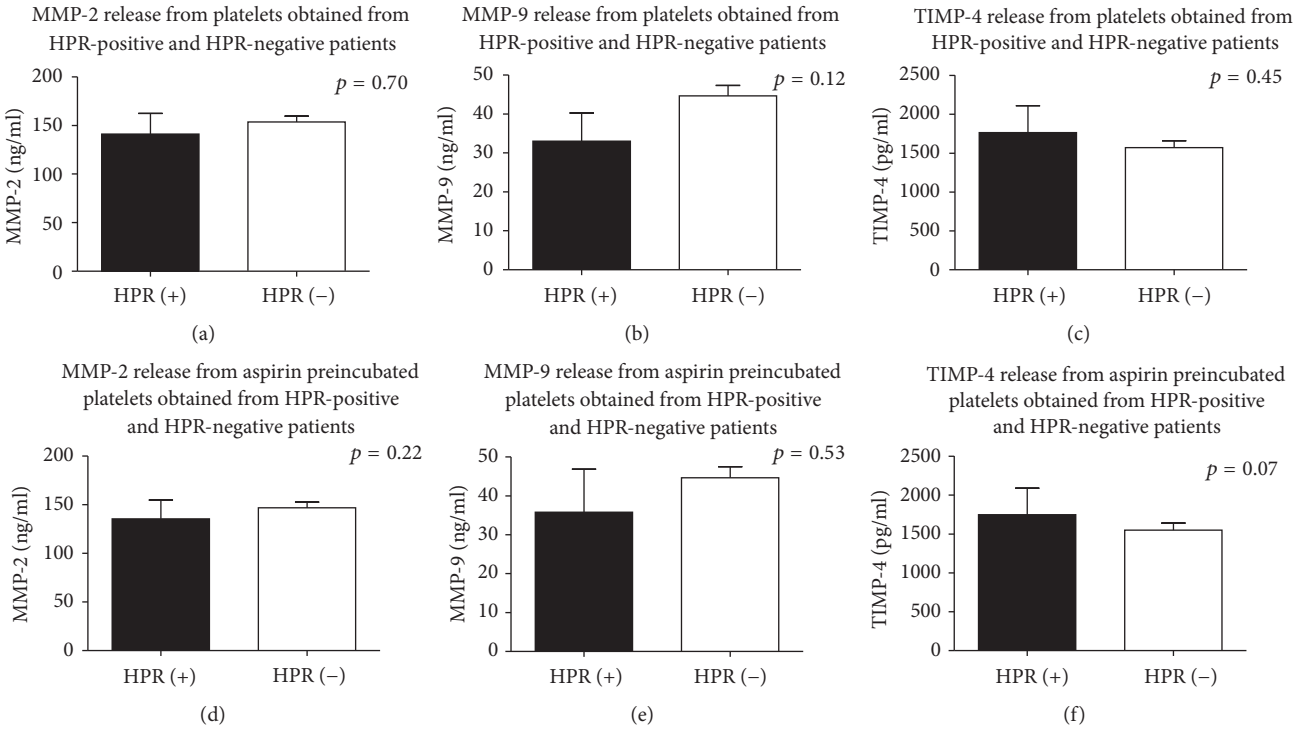

Figure 2: An influence of HPR on platelets release of MMP-2, MMP-9, and TIMP-4 without (a–c) or with (d–f) pretreatment with parenteral form of aspirin. Mean ± SEM classification of HPR on the basis of LTA Amax > 20%. HPR-high on-aspirin treatment platelets reactivity; LTA: light transmittance aggregometry; MMP: matrix metalloproteinase; TIMP-4: tissue inhibitor of MMPs.

3.4. MMPs and TIMP-4 in Patients with or without Diabetes. DM-positive and DM-negative patients were verified in respect of MMP-2, MMP-9, and TIMP-4 concentration in plasma and supernatants after platelets aggregation. Diabetics with CAD showed an increased plasma concentration of MMP-2 as well as an increased MMP-2 in supernatants after platelets aggregation in comparison to CAD patients without diabetes (Table 3, Figure 4(a)). However, DM had no influence on MMP-9 and TIMP-4 concentrations in plasma and their release from platelets during aggregation (Table 3, Figures 4(b) and 4(c)).

Interestingly, although the presence of HPR had no influence on plasma level of MMP-2 as well as its release from activated platelets (as indicated above), diabetic subjects with HPR (meeting MEA criteria) showed an increased plasma MMP-2 in comparison to diabetics without HPR (213.6 ± 76.1 versus 164.8 ± 54.6, borderline significance $p = 0.052$).

4. Discussion

Acetylsalicylic acid is widely used in clinical practice during coronary interventions and in long-term secondary prevention of cardiovascular and cerebrovascular events. Unfortunately, in some patients, higher than expected platelet reactivity during antiplatelet therapy (a high on-treatment platelet reactivity, HPR) is observed. The limited inhibition of platelet function due to incomplete inactivation of cyclooxygenase 1 enzyme (COX-1) [27, 28] is associated with poor cardiovascular outcomes [29]. Sawicki et al. (1997) showed that MMP-2 is released from platelets during platelets stimulation and facilitates their aggregation by non-ADP and non-TXA2 pathway [25, 26]; building upon this finding, some contrary results describing an influence of aspirin on MMPs/TIMP pathway have been published. Falcinelli et al. (2007) and others showed that treatment with aspirin did not affect the translocation and release of MMP-2 from platelets into plasma [30, 31], but Hua et al. (2009) reported that aspirin decreases an expression and release of MMP-2 and MMP-9 from monocytes [32]. Others showed that MMP-9 can influence the action of aspirin through modification of the TXA_2 pathway [23] and that aspirin can influence MMP-2 and MMP-9 production in monocytes [32] and megakaryocytes [33, 34].

In our study we observed that plasma concentration of MMP-2 in patients with high on-aspirin treatment platelets reactivity was similar to those with proper response to aspirin (HPR-negative). This suggests that the concentration of plasma MMP-2 was not associated with an increased aggregation of platelets in HPR-positive patients. Following the previous hypothesis that MMP-2 is released from platelets during their aggregation and potentiates platelets aggregation [25], we explored whether decreased susceptibility of platelets to aspirin treatment affects release of MMP-2 in vivo. Data showed that platelets with lower response to aspirin released as much MMP-2 as platelets with total inhibition of COX1. This means that inhibition of COX1/COX2 in platelets of patients treated with aspirin has little impact on MMP-2 release. Moreover, indirect inhibition of platelets

Table 3: MMP-2, MMP-9, and TIMP-4 level in diabetes and nondiabetes subjects.

	DM present and CAD present ($n = 35$)	DM absent and CAD present ($n = 35$)	Statistical significance
Plasma level			
MMP-2 (ng/ml)	176.3 ± 5.5	134.0 ± 3.8	$p < 0.001$
MMP-9 (ng/ml)	50.2 ± 4.6	42.9 ± 4.2	NS
TIMP-4 (pg/ml)	1621.9 ± 134.4	1622 ± 123.4	NS
Supernatant after LTA induced by AA			
MMP-2 (ng/ml)	174.7 ± 9.8	132.7 ± 4.0	$p < 0.001$
MMP-9 (ng/ml)	47.9 ± 3.2	40.6 ± 3.9	NS
TIMP-4 (pg/ml)	1569.8 ± 127.4	1584.1 ± 117.9	NS
Supernatant after 5 minutes of aspirin incubation and subsequent LTA induced by AA			
MMP-2 (ng/ml)	168.2 ± 9.5	125.9 ± 3.7	$p < 0.0001$
MMP-9 (ng/ml)	49.2 ± 3.6	39.8 ± 3.8	NS
TIMP-4 (pg/ml)	1550.2 ± 129.4	1563.3 ± 119.8	NS

Notes. AA: arachidonic acid, CAD: coronary artery disease, DM: diabetes mellitus, HPR: high on-treatment platelet reactivity, LTA: light transmittance aggregometry, MMP-2: matrix metalloproteinase-2, MMP-9: matrix metalloproteinase-9, MEA: multielectrode aggregometry, TIMP-4: tissue inhibitor of matrix metalloproteinase-4, NS: statistically not significant, TXB2: thromboxane B2; mean ± SEM.

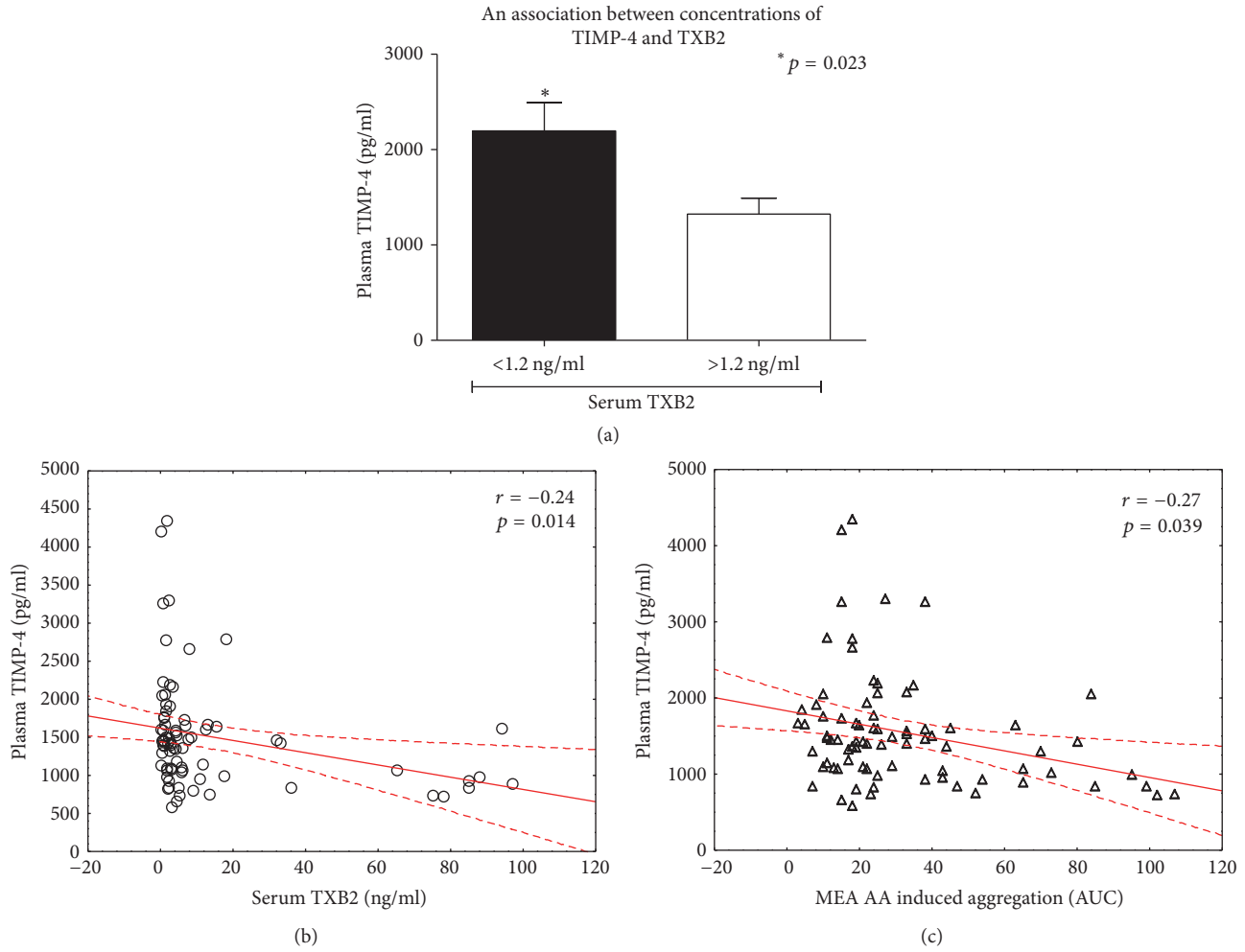

Figure 3: An association of TIMP-4 with TXB2 (a-b) and platelets aggregation (c). Mean ± SEM; platelets aggregation tested by MEA. MEA: multielectrode aggregometry; TXB2: thromboxane B2; TIMP-4: tissue inhibitor of MMP.

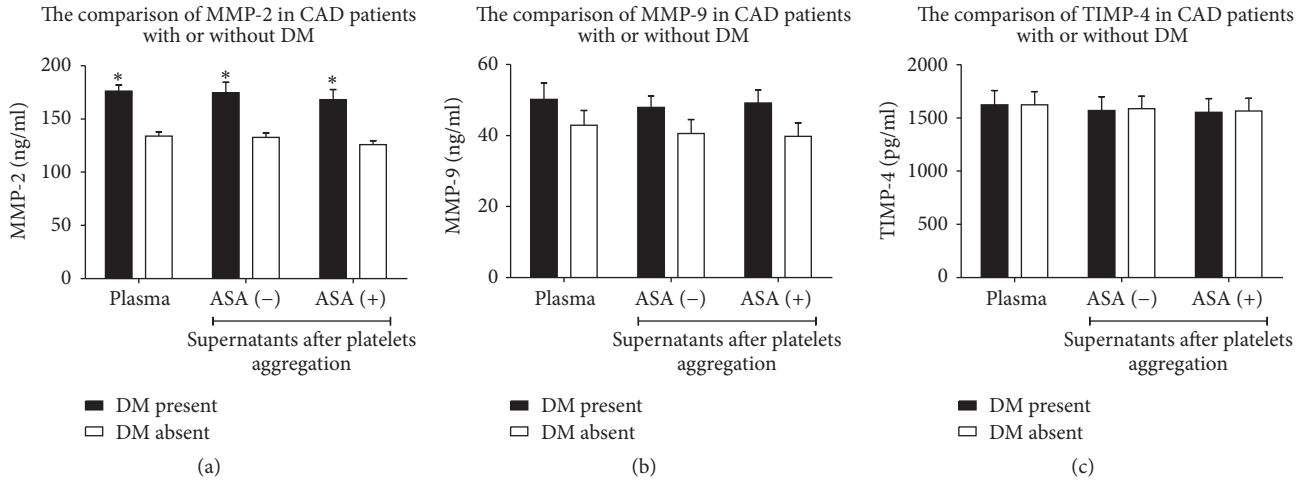

Figure 4: The comparison of MMP-2 (a), MMP-9 (b), and TIMP-4 (c) concentrations in plasma and supernatants after platelets aggregation in patients with or without diabetes mellitus (DM). Mean ± SEM. ASA: aspirin; CAD: coronary artery disease; DM: diabetes mellitus; MMP-2: matrix metalloproteinase 2; MMP-9: matrix metalloproteinase 9; TIMP-4: tissue inhibitor of MMPs; $^{*}p < 0.001$.

with aspirin before its stimulation with arachidonic acid (AA) did not show a significant effect on MMP-2 release. Therefore, other COX-mediated mechanisms associated with platelets release of metalloproteinases likely exist, or platelets excretion of MMP-2 has little or no impact on total plasma concentration of MMP-2 in vivo. Falcinelli et al. (2007) also showed that administration of aspirin did not significantly affect the surface expression and release of MMP-2 from platelets activated by vessel damage or TRAP [30]. Although previous in vitro studies showed that MMP-2 is released by platelets upon activation, nonphysiological stimuli (very high concentrations of thrombin) were used and were not affected from physiological factors regulating platelets activation in vivo [25]. Because platelets activated with AA in our in vivo study did not increase the total amount of plasma MMP-2, this may also suggest that plasma MMP-2 is mainly subjected to MMP-2 released from other blood or tissue cells.

In the previous studies, it was shown that platelet release of MMP-2 was triggered by collagen induced aggregation [24] while its release upon thrombin was controversial [36]. This is the first report focusing on MMP-2 release from platelets activated by arachidonic acid (AA). Taking into account the fact that there was no difference in the platelets' release of MMP-2 in our study, it is very likely that arachidonic acid affects platelets' excretion of MMP-2 independently of COX-1/COX-2 inhibition by aspirin. A study by Hermann et al. (2001) supports this hypothesis by showing that translocation of cytosol proteins into platelets' surface during aggregation is resistant to aspirin inhibition [37].

MMP-9 is another platelets-derived matrix metalloproteinase [38]. The antiaggregatory role of MMP-9 based on TXA2 dependent pathway and independent of stimulator of aggregation has been well documented [23, 38, 39]. Both MMP-9 and MMP-2 were shown to be released from platelets into coronary circulation during acute coronary syndrome, showing the potential association of MMPs and the development of acute coronary syndrome [30]. Although the potential for acetylation of serine at the catalytic site of COX-1/COX-2 by aspirin inhibits the release of matrix metalloproteinases from platelets [40], we did not show an influence of aspirin on MMP-9 excretion and on its anti-aggregatory function in subjects with proper or insufficient responsiveness to aspirin. Moreover, plasma concentration of MMP-9 was slightly decreased in patients with high on-aspirin treatment platelets reactivity, suggesting that the response of platelets to aspirin treatment in HPR patients is opposite to our previous hypothesis. Potentially, HPR should lead to an increased expression and release of platelet MMPs due to incomplete inhibition of COX-1/COX-2 pathway. Here, we observed slightly decreased plasma concentration of MMP-9 in HPR-positive patients and no MMP-9 release from platelets upon stimulation with arachidonic acid. Previous studies reported contrary data about the release of MMP-9 upon stimulation with collagen, thrombin, and TLR2 agonist Pam3CSK4 [36, 39]. The same authors suggested that presence of MMP-9 in peripheral blood was attributed mainly to neutrophil release [36]. Some investigators are even skeptical of the presence of MMP-9 in platelets [41, 42].

Higher mortality due to acute myocardium infarction is observed in diabetic patients than in nondiabetic subjects [2]. An increased propensity of atherosclerotic plaques to ulceration and thrombosis in diabetics enhanced a risk of fatal outcome [43]. Regulation of MMPs in DM has been widely investigated. Hyperglycemia increases an expression and activity of MMP-2 and MMP-9 in aortic smooth muscle cells, vascular tissue, and plasma [4, 44], affecting metabolism of the extracellular matrix. Studies suggest that hyperglycemia increases oxidative stress in various cells, leading to an activation of COX-2 which in turn induces a biosynthesis of MMP-2 and MMP-9 [45, 46]. Schulze et al. (2003) reported that imbalance between MMPs and TIMPs results in enhanced MMP activity and then in several cardiovascular disorders [47]. Because MMPs participate in rupture of atherosclerotic plaques [48], we decided to

check whether chronically elevated plasma glucose increases the expression and release of MMP-2 and MMP-9, hence accelerating the risk for acute coronary syndromes. We showed that diabetic patients with coronary artery disease presented significantly higher plasma levels of MMP-2, but not MMP-9, than nondiabetic counterparts. We also exclude that other factors such as arterial hypertension, history of myocardial infarction, and hypercholesterolemia had an influence on MMP-2 level in DM patients (Table 1). Our results are consistent with previous reports showing that hyperglycemia is associated with increased plasma concentration of MMP-2 [49–51]. We suspect that nonsignificant increase of MMP-9 resulted from the small number of tested subjects. However, data from Baugh et al. (2003) revealed no significant difference in MMP-9 production in DM [52], and data from Bhatt and Veeranjaneyulu (2014) indicated that MMP-2 level was highly elevated in comparison to MMP-9 [53]. Matrix metalloproteinases are regarded to be the key molecules in inflammation [10, 54]. They are implicated in the accumulation of inflammatory cells, healing of tissue injury, and remodeling processes [55, 56]. Taking into account the fact that diabetes mellitus is a chronic inflammatory disease, an induction of proinflammatory factors leads in consequence to recruitment of monocytes, macrophages, and granulocytes, which are a rich source of MMPs. Since all participants of our study group had coronary artery disease and only those suffering from DM had elevated plasma MMP-2, an important observation is that DM was a strong determinant of increased plasma MMPs. However, taking into account the fact that plasma concentration of MMP-2 was also elevated in the group of diabetic subjects with aspirin-HPR with respect to diabetics without aspirin-HPR and high on-aspirin treatment platelets reactivity did not increase plasma MMP-2 irrespective to diabetes mellitus, this suggests that coincidence of both DM and HPR may affect plasma level of MMP-2.

Physiologically, matrix metalloproteinase activity in the extracellular matrix is regulated by the family of tissue inhibitors of matrix metalloproteinases (TIMPs). Because gelatinases bind TIMPs to form a tightly bound 1 : 1 molar stoichiometric complex [57], an increased expression of MMP-2 should be accompanied by enhanced production of TIMPs [50]. Since TIMP-1 and TIMP-2 may also be present in platelets [58], it was shown that TIMP-4 is the major intraplatelet inhibitor of MMPs [26]. Moreover, it was demonstrated that TIMP-4 and MMP-2 colocalized in resting platelets and upon aggregation by aggregating agents such as collagen and thrombin MMP-2 is translocated to the platelet surface and TIMP-4 is released to plasma. Hence, the dissociation of TIMP-4 from its complex with MMP-2 may facilitate the interactions of MMP-2 with its receptors and stimulate aggregation [24]. However, an increased plasma concentration of MMP-2 in diabetics of our study was not accompanied by increased plasma concentration of TIMP-4. This suggests that hyperglycemia affects the physiological production of TIMP-4 following increased MMP-2 synthesis. Radomski et al. (2002) reported that TIMP-4 is colocalized with MMP-2 in resting platelets and is released from platelets upon aggregation [26]. Hence we hypothesized that the dissociation of TIMP-4 from TIMP-4-MMP-2 complex may stimulate platelets aggregation due to interactions of MMP-2 with its receptors on platelets. Also in this case our study did not confirm that dissociation of TIMP-4 and MMP-2 from their complex led to enhanced release of MMP-2/TIMP. It is most likely that enhanced concentrations of plasma MMP-2 in DM subjects were caused by stimulation of cells other than platelets.

Although we did not notice a significant in vitro release of TIMP-4 from platelets after stimulation with arachidonic acid and there was no association between aspirin-HPR and plasma level of TIMP-4, we showed that plasma concentration of TIMP-4 was the highest in patients with the lowest TXB2 level. Together with the negative correlation between TIMP-4, TXB2, and platelets aggregation, this could imply TIMP-4 as a marker of low on-treatment platelet reactivity.

In conclusion, our pilot study observed that administration of aspirin for one week before blood collection from aspirin-HPR-positive and aspirin-HPR-negative subjects was not associated with translocation and release of both matrix metalloproteinases and its selective inhibitor from platelets into plasma. Also direct treatment of platelets with ASA did not affect platelets release of MMPs/TIMP-4 during activation and aggregation induced by arachidonic acid. The contribution of MMP-2, MMP-9, and TIMP-4 in the regulation of platelets function in patients with high on-aspirin treatment platelets reactivity is negligible and decreased plasma concentration of TIMP-4 may serve as a marker of TXA2-mediated platelets aggregation. It is also worth noting that chronically elevated plasma glucose increases plasma concentration of MMP-2, and aspirin-HPR potentiates this phenomenon. For this reason, an inhibition of MMP-2 with selective inhibitors should be considered for the prevention of DM-induced cardiovascular complications.

Additional Points

Study Limitations. The study group is rather small but the results, especially those obtained in vitro, are consistent. Data represents a pilot study. In previous reports, MMPs and TIMP-4 were shown to be released upon platelet activation with collagen and to some extent thrombin. We used arachidonic acid as an agonist of aggregation, because (a) it is widely accepted to monitor aspirin response [28], (b) MMPs/TIMP release from platelets was not tested after arachidonic acid stimulation so far, and (c) this agonist was chosen to assess aspirin effect and possible MMPs/TIMP interaction. We measured MMPs/TIMP plasma concentration, not activity, because the use of zymography which allows for measurement of activity of both active and nonactive forms of enzymes (proenzymes and enzymes) seems doubtful.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Wiktor Kuliczkowski, Mariusz Gąsior, Joanna Urbaniak, Jacek Kaczmarski, and Iwona Bil-Lula performed the

research; Wiktor Kuliczkowski, Marek Radomski, and Andrzej Mysiak designed the experimental part of the project; Marek Radomski and Marta Negrusz-Kawecka provided the clinical expertise; Wiktor Kuliczkowski and Iwona Bil-Lula analyzed the data; Iwona Bil-Lula and Wiktor Kuliczkowski wrote the article.

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Research Article

Effects of Hyperglycemia on Vascular Smooth Muscle Ca^{2+} Signaling

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Diabetes is a complex disease that is characterized with hyperglycemia, dyslipidemia, and insulin resistance. These pathologies are associated with significant cardiovascular implications that affect both the macro- and microvasculature. It is therefore important to understand the effects of various pathologies associated with diabetes on the vasculature. Here we directly test the effects of hyperglycemia on vascular smooth muscle (VSM) Ca^{2+} signaling in an isolated in vitro system using the A7r5 rat aortic cell line as a model. We find that prolonged exposure of A7r5 cells to hyperglycemia (weeks) is associated with changes to Ca^{2+} signaling, including most prominently an inhibition of the passive ER Ca^{2+} leak and the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA). To translate these findings to the in vivo condition, we used primary VSM cells from normal and diabetic subjects and find that only the inhibition of the ER Ca^{2+} leaks replicates in cells from diabetic donors. These results show that prolonged hyperglycemia in isolation alters the Ca^{2+} signaling machinery in VSM cells. However, these alterations are not readily translatable to the whole organism situation where alterations to the Ca^{2+} signaling machinery are different.

1. Introduction

Diabetes is a complex multifactorial disease characterized by the onset of dyslipidemia, early hyperinsulinemia, and hyperinsulinemia, followed by pancreatic β-cell failure leading to hyperglycemia and insulin resistance [1–3]. This imbalance is associated with long-term complications and injury to multiple particularly susceptible organ systems, including the eye (retinopathy), kidney (nephropathy), peripheral nervous system (neuropathy), heart, and the vasculature (cardiovascular disease) [4]. There is significant evidence in the literature in both humans and animal models in support of the hypothesis that these pathologies are at least in part associated with the lack of glycemic control [4–6]. However, in addition to hyperglycemia, diabetes is associated with dyslipidemia, hyperinsulinemia, and increase reactive oxygen species [7]. This makes assignment of complications to a specific dysregulation at the whole organism level problematic.

Morbidity and mortality of individuals with diabetes result mainly from vascular dysfunction (VD) [3, 8–11]. Vascular complications associated with diabetes are divided into macrovascular complications, which include atherosclerosis, coronary artery disease, peripheral vascular disease, and microvascular complications such as retinopathy, nephropathy, and neuropathy [12–14]. Endothelial dysfunction plays an important role in vascular complications during diabetes [7]. Although in diabetes the mechanism of VD is complex and multifactorial involving multiple pathways [9, 13, 15, 16], chronic hyperglycemia is an important contributor to this process [3, 9, 10, 17].

Vascular smooth muscle (VSM) cells, existing in a differentiated quiescent state in the blood vessel wall, have a large repertoire of ion channels, receptors, signaling molecules, and contractile proteins essential for their contractile function [18, 19]. Because VSM contraction is dependent on a rise of cytoplasmic Ca^{2+} levels changes in VSM Ca^{2+} signaling have significant impact on determining vascular tone and peripheral resistance as both are dependent on resistance arteries diameter [20]. Consequently, any damage/modifications in the activity of key players involved in Ca^{2+} homeostasis are likely to be associated with VD. VSM cells play a key role in hyperglycemia-induced VD, including hypertension. Several lines of evidence suggest that oxidative stress caused by hyperglycemia provokes molecular

pathologies that contribute to VD, leading to increased risk of adverse cardiovascular disease associated with diabetes [3, 10–12, 21–28]. Reactive oxygen species (ROS) are instrumental regulators of intracellular Ca^{2+} homeostasis and influence several other intracellular signaling pathways [29, 30]. Even though ROS generation is highly controlled in the vasculature, under physiological conditions, an increase in ROS generation under pathologic conditions contributes to vascular damage and cardiovascular disease [29]. Hyperglycemia-induced ROS is considered an important link between hyperglycemia and pathways of diabetic-related vascular complications [8, 16]. This is due to the fact that hyperglycemia-induced ROS is capable of damaging DNA and proteins and of inducing lipid peroxidation [23, 29]. This latter pathology affects ion transport across the cell membrane through two possible mechanisms: (1) inducing nonspecific leak of ions through the lipid bilayer and/or (2) modifying the physical properties of phospholipids in a way that alters the function of channels, pumps, and exchangers that are embedded within the lipid bilayer [29, 30]. For instance, inhibition of the sarcoplasmic/endoplasmic reticulum ATPase (SERCA) pump by hyperglycemia-induced ROS production results in an increase in intracellular Ca^{2+} [10]. This increase plays a role in the pathogenesis of vascular dysfunction by enhancing VSM cell migration [9, 10, 15]. The rat aortic cell line, A7r5, is a useful model for studying the effects of hyperglycemia on VSM function especially in the context of Ca^{2+} signaling since these cells have the complement of Ca^{2+} channels and pumps activities observed in freshly dispersed VSM cells [20, 31–33]. Searls et al. showed that in contrast to the defects observed in diabetic mice Ca^{2+} signaling pathways in A7r5 cells were not affected when the cells were shifted for short term from the physiological 5 mM glucose to the glycemic 25 mM and supraphysiological glucose 75 mM concentration was needed to see a significant effect [34].

Based on these findings our goal from the present work was to investigate the effect of prolonged exposure to high glucose levels on Ca^{2+} homeostasis in the A7r5 VSM cell line to better understand the potential pathology of hyperglycemia on the Ca^{2+} signaling machinery of VSM and potential implications on cardiovascular disease.

2. Materials and Methods

2.1. Materials. Dulbecco's modified Eagle's medium (DMEM), sodium pyruvate, penicillin/streptomycin, fetal bovine serum (FBS), phosphate-buffered saline (PBS), Trypsin/EDTA, N-acetyl-cysteine (NAC), mannitol, and phenylephrine (PE) were from Sigma Aldrich, St. Louis, MO, USA. Dimethylsulfoxide (DMSO) was obtained from Amresco (Amresco, USA). Thapsigargin (TG) was from Invitrogen and ionomycin was from Life Technologies.

2.2. Cell Culture. The embryonic rat aortic smooth muscle A7r5 cells (ATCC, Manassas, Virginia, USA) were grown, as recommended, in DMEM-high glucose (HG: 4.5 g/l equivalent to 25 mM), supplemented with 1% penicillin/streptomycin, 1% sodium pyruvate, and 10% FBS. To test the effect of glucose on the proliferation, metabolic activity, and Ca^{2+} signaling pathways in A7r5 cells, cells already cultured in HG (25 mM glucose) were shifted to DMEM-normal glucose (NG: 1 g/l equivalent to 5.5 mM) for more than 4 weeks. To rule out the osmotic effect induced by glucose, cells cultured in NG were shifted, for more than 4 weeks, to NG supplemented with mannitol (19.5 mM), a nonmetabolizable sugar. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Throughout the manuscript, cells cultured under DMEM-HG, DMEM-NG, and DMEM-NG with mannitol are refereed to, respectively, as HG, NG, and OC streams. Primary human, aortic smooth muscle cells, from normal (NHVSMC) and diabetic (DHVSMC) individuals, were obtained from Lonza Walkersville (Walkersville, MD). Cells were grown, as recommended in smooth muscle basal medium supplemented with 5% FBS and a cocktail of different cytokines and growth factors obtained from Lonza Walkersville (Walkersville, MD). ReagentPack™ Subculture (kits including Trypsin/EDTA, Trypsin neutralizing solution, and HEPES buffered saline) designed specifically for the passaging of primary cell types was obtained from Lonza Walkersville (Walkersville, MD). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

2.3. Cell Proliferation. The proliferation of A7r5 cells was determined using trypan blue dye exclusion counting using TC10 automated cell counter (Bio-Rad, CA, USA).

2.4. WST-1 Metabolic Activity Assay. To test whether the concentration of glucose affects the metabolic activity of the cells, A7r5 cells from HG, NG, and OC streams were plated, at equal density, in 96-well plates and shifted to HG or NG according to the followings: (NG:NG, NG: shifted to HG); (HG:HG, HG: shifted to NG), and (OC:OC, OC: shifted to HG). Three hours after plating the metabolic activity of the cells was evaluated by using the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulfonate) assay (Roche Diagnostics GmbH, Mannheim, Germany). Using this assay, the ability of the cells to cleave by mitochondrial dehydrogenases the WST-1 tetrazolium salt to the red colored formazan allows assessing the metabolic activity of the cells under different glucose concentration. The absorbance was measured at 440 nm using Envision 2104 Multilabel Reader (Perkin Elmer, Massachusetts, USA).

2.5. Intracellular Calcium Measurements. A7r5 cells were cultured on 35 mm poly-d-lysine coated glass coverslips (MatTek corp, MA) and incubated in their respective media at 37°C. When 60–70% confluent, A7r5 cells were loaded with 2 μM Fura-2AM (Invitrogen, NY, USA) for 30 min at 37°C. After incubation, cells were washed with PBS and incubated with Ca^{2+} containing Ringer buffer for 10 min at room temperature (RT) before analysis. The Ca^{2+} containing Ringer buffer contained (in mM) 120 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 2.0 $CaCl_2$, 5.5 glucose, and 20 HEPES (pH 7.4). In Ca^{2+} free Ringer buffer, $CaCl_2$ was replaced with equimolar $MgCl_2$. For experiments that required low Na^+, Ringer buffer NaCl was replaced with equimolar amount of N-methyl-D-glucamine ($NMDG^+$).

The imaging system included inverted epifluorescence microscope (Olympus IX71, PA) connected to a CoolSNAP HQ2 charged coupled device (CCD) camera. Image acquisition was performed using EasyRatioPro calcium imaging system (PTI, NJ).

Changes in cytosolic Ca^{2+} level were determined from the ratio of Fura-2AM fluorescence emission intensities following excitation at 340 and 380 nm.

2.6. Vascular Smooth Muscle Cells Contraction. Assessment of cell contraction was performed on A7r5 cells cultured on glass coverslips. Cells were washed with prewarmed Ca^{2+}/Mg^{2+}-free PBS and incubated in HBS for 10 min prior to live cell imaging. Cell contraction was visualized using an inverted microscope (Olympus, Japan) equipped with a LucPlan FLN 40x/0.60 objective. Images were acquired with a CCD camera (Olympus DP72, Germany) and processed using DP2-BSW software (Olympus Soft Imaging Solutions, Germany). Cell contraction was quantified by morphometric analysis using NIH software (ImageJ). Briefly, addition of 20 mM KCl induces the formation of contractile fibers that appears as protruded edges on the surface of contracted cells. To quantify contractile responses to KCl, contractile fibers were quantified using the edge detection function in ImageJ on thresholded time-lapse images.

2.7. Intracellular ROS Generation by DCFH. To test whether cells cultured under HG have higher reactive oxygen species (ROS) level than those cultured in NG and OC media, the level of ROS in cells cultured in media with different glucose concentration was examined using 2′,7′dichlorodihydrofluorescein diacetate (DCFH-DA) (Acros Organics, New Jersey, USA). In this assay, the DCFH-DA molecule passively diffuses into the cells and is cleaved and oxidized in the intracellular environment by ROS to the green fluorescence emitting compound, 2′,7′-dichlorofluorescein (DCF). Briefly, cells from HG, NG, and OC streams were plated, at equal density, in 96-well plates and after overnight starvation in 1% FBS, cells were incubated with 100 μM DCFH-DA prepared in PBS at 37°C. 30 min after incubation, cells were shifted to HG, NG, or OC for an additional 30 min. Hydrogen peroxide (H_2O_2) (Sigma Aldrich, St. Louis, MO, USA) was used at 250 μM as a positive control. DCF fluorescence was then determined, after cells lysis in 90% DMSO/10% PBS for 10 min in the dark, using a fluorescent plate reader (Envision 2104 Multilabel Reader, PerkinElmer, Massachusetts, USA) with 485 nm excitation and 520 nm emission wavelengths.

2.8. Western Blot Analysis. Total protein from HG, NG, and OC streams was extracted using RIPA buffer (Sigma Aldrich, St. Louis, MO, USA) supplemented with phosphatase and protease inhibitors. Protein extracts were quantified using Bradford method using Bio-Rad Protein Assay Dye Reagent (Bio-Rad, CA, USA) according to the manufacturer's protocol. Protein samples were mixed, respectively, with 10% and 25% of reducing agent and LDS buffer containing bromophenol blue for gel electrophoresis (Invitrogen, Carlsbad, CA, USA). An equal amount of protein lysate was subjected to gel electrophoresis on NuPAGE Bis-Tris or Tris-Acetate Gels (Invitrogen, Carlsbad, CA, USA) for 50 min at 200 V. Proteins were then transferred to a PVDF transfer membrane (Kisher Biotech, Germany) at 30 V for 30 min. After transfer, membranes were immunoblotted with the following primary antibodies: PMCA1, PMCA 4, and PMCA total (Affinity Bioreagents, Rockford, IL), SERCA 2 (Thermo Scientific, Pierce antibodies, Rockford, IL, USA), NCX1 (Swant, Bellinzona, Switzerland), STIM1 (Cell Signaling, Beverly, USA), Orai1 (Proteintech, USA), and IP3R1 (Millipore, USA). β-Actin, α-tubulin, and the secondary antibodies were from Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA). Following incubation with secondary antibodies, membranes were reacted with enhanced chemiluminescence western blot detection reagent (Amersham, GE Healthcare, UK). The luminescent reactivity was then measured using an image acquisition system, Gene SNAP, Geliance 600 Imaging system (Perkin Elmer, Massachusetts, USA). All membranes were stripped with Restore Plus Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL, USA) and equal loading was then verified through reprobing the membranes with ß-actin/α-tubulin. Further densitometry analysis was performed using Gene Tools, Geliance 600 Imaging system (Perkin Elmer, Massachusetts, USA).

2.9. RNA Extraction and Real-Time PCR. RNA was extracted from A7r5 cells cultured in HG, NG, and OC using Qiagen RNAeasy extraction kit (Hilden, Germany) and reversed transcribed using High-Capacity cDNA reverse transcription kit (Applied Biosystems (AB)), all following the manufacturer's instructions. Quantitative real-time reverse transcriptase (qRT-PCR) was used to analyze the expression of the following genes (PMCA1, PMCA4, SERCA1, and SERCA2) using 2 μL of template cDNA. α-Actin was used as housekeeping gene. Quantitect primers were obtained from Qiagen (Hilden, Germany). qRT-PCR was performed with the Fast SYBR Green Master Mix (2x) according to the manufacturer's instructions. Each PCR generated only the expected amplicon as shown by the negative first-deviation plots of the melting curve. Results were normalized to non-induced housekeeping gene-levels. Samples were analyzed in duplicate from three independent experiments.

2.10. Statistical Analysis. Results are presented as mean ± standard error of the mean (SE) from at least three independent experiments done each in triplicate. Comparison between the different groups was done using Nonparametric/Kruskal-Wallis test using IBM SPSS Statistics 23 software. The level of significance was set at 0.05.

3. Results

3.1. Long-Term Adaptation of A7r5 to Low or High Glucose Culture Conditions. A7r5 rat aortic smooth muscle cells are typically cultured in high glucose concentration of 25 mM. This concentration is hyperglycemic (HG) as the level of glucose in the blood of diabetic rats is around 17 mM, in contrast to the normoglycemic (NG) levels of 5.5 mM [34]. To begin to characterize the effect of hyperglycemia on

Ca^{2+} signaling in VSM cells, we switched A7r5 cells initially cultured in HG to NG for up to 72 h and tested various Ca^{2+} signaling modalities, including basal Ca^{2+} levels, store-operated Ca^{2+} entry (SOCE), Ca^{2+} decay, and Ca^{2+} release but could not detect any alterations in Ca^{2+} signaling that correlate with the medium glucose concentrations (Supplemental Data, Figure 1, in Supplementary Material available online at https://doi.org/10.1155/2017/3691349). This data is in accordance with the study by Searls et al., who failed to show significant effect on Ca^{2+} when glucose levels were switched for a short time between 5 and 25 mM, prompting them to switch to 75 mM for short-term studies to replicate effect observed in diabetic mice [34].

Because complications associated with diabetes manifest themselves over prolonged periods of time (years in humans), we were interested in determining whether long-term exposure of VSM cells to HG concentrations affects Ca^{2+} signaling. We therefore incubated A7r5 cells for a minimum of 4 weeks in physiological glucose (5.5 mM) to mirror normoglycemia (NG) in the animal. Once cells were switched to the NG stream they were maintained under these culture conditions for the duration of the study. Alternatively another stream of A7r5 cells was cultured in 25 mM glucose representing the hyperglycemic group (HG). In addition, an osmotic control for the HG treatment was included (OC), where NG was supplemented with 19.5 mM mannitol, to separate effects due to hyperglycemia from those resulting from changes due to osmolarity at the high glucose concentrations.

Surprisingly, long-term incubation of A7r5 cells in NG or HG does not significantly alter their proliferation rate (Figure 1(a)). This is likely due to the adaptation of the cells to the level of glucose used. In contrast, switching cells to different glucose concentrations results in small but detectable and statistically significant differences in their metabolic activity (Figure 1(b)). When A7r5 cells were shifted from NG or OC streams to HG for 3 h, this was associated with higher metabolic activity ($p < 0.05$) as compared to their respective controls. Similarly, when A7r5 cells were transferred from the HG stream to NG, they exhibited lower metabolic activity ($p < 0.05$). Furthermore, long-term incubation of A7r5 cells under the different streams NG, HG, or OC does not alter their basal resting cytoplasmic Ca^{2+} levels measured using Fura-2AM in Ca^{2+}-free or Ca^{2+}-containing extracellular solution (Figure 1(c)). We also measured intracellular store Ca^{2+} content in A7r5 cells cultured in the different streams using the ionomycin releasable Ca^{2+} pool in Ca^{2+}-free media as illustrated in Figure 1(d). Ionomycin is a Ca^{2+} ionophore that is inserted into the lipid bilayer and nonspecifically equilibrates Ca^{2+}. As such in Ca^{2+}-free media, it releases Ca^{2+} trapped in intracellular stores such as the endoplasmic reticulum (ER). Incubating A7r5 cells in different glucose concentrations for extended time periods does not alter intracellular Ca^{2+} store content (Figures 1(d) and 1(e)).

Phenylephrine (PE) is an α-adrenergic receptor agonist that couples to trimeric G-proteins to activate phospholipase C (PLC) and generate inositol 1,4,5-triphosphate receptor (IP_3). IP_3 in turn binds to IP_3 receptors (IP_3Rs) on the ER membrane to gate them open and release intracellular Ca^{2+}. The maximum levels of PE-induced Ca^{2+} release in cells cultured under the different glucose conditions were not statistically different arguing that the signal transduction cascade downstream of PE is not altered significantly when cells are cultured under hyperglycemia conditions (Figures 2(a) and 2(b)). Therefore, dramatic changes in the activity of the α-adrenergic receptor, G-proteins, or PLC are unlikely. Furthermore, protein levels of the IP_3Rs are similar among the three tested streams (NG. HG, and OC) (Figure 2(c)), showing that glucose levels do not affect IP_3Rs expression levels.

3.2. Store-Operated Ca^{2+} Entry (SOCE). Agonist-mediated release of intracellular Ca^{2+} results in store depletion and the activation of a ubiquitous Ca^{2+} influx pathway known as store-operated Ca^{2+} entry (SOCE) [35]. This pathway is activated through the consorted action of two essential molecules: Orai1, a highly selective Ca^{2+} channel at the plasma membrane, and STIM1, a single pass ER membrane protein [36, 37]. The luminal domain of STIM1 has an EF-hand motif that senses Ca^{2+} store content [38, 39]. Ca^{2+} release in response to agonist stimulation lowers Ca^{2+} store content, which results in STIM1 oligomerization and its translocation to subplasma membrane cortical ER domains; where it recruits Orai1 and gates it open allowing Ca^{2+} influx [40–44]. Consequently, SOCE maintains Ca^{2+} homeostasis by ensuring the replenishment of the ER Ca^{2+} stores following store depletion.

To evaluate the effect of glucose on SOCE in A7r5 cells, we measured SOCE levels using the classical Ca^{2+} readdition protocol after store depletion. For this assay Ca^{2+} stores were depleted with thapsigargin (TG), a specific nonreversible inhibitor of the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) that blocks store refilling and leads to store depletion due to an ill-defined continuous Ca^{2+} leak from the ER. Store depletion was performed in Ca^{2+}-free conditions after which cells were switched to Ca^{2+}-containing medium, which results in Ca^{2+} influx mediated by SOCE (Figure 3(a)). Incubation of A7r5 cells under hyperglycemic conditions results in a small but statistically significant decrease in SOCE ($p < 0.034$) (Figures 3(a) and 3(b)). A similar decrease in SOCE levels has been also observed in cells cultured under high osmolarity (OC) ($p < 0.053$), which mimic the osmotic conditions resulting from increased glucose levels in HG medium (Figure 3(b)). This argues that inhibition of SOCE under HG conditions is independent of glucose and likely due to the increased osmolarity.

We further tested the expression levels of the two primary SOCE proteins Orai1 and STIM1. Western blot analysis of A7r5 cells cultured under NG, HG, or OC shows that the expression levels of both STIM1 (Figure 3(c)) and Orai1 (Figure 3(d)) are not significantly different between the three groups. These results argue that extracellular glucose concentrations do not affect SOCE protein expression levels or SOCE activity.

3.3. Voltage Gated Ca^{2+} Channels (VGCC). It is well established that Ca^{2+} entry through VGCCs, specifically the L-type

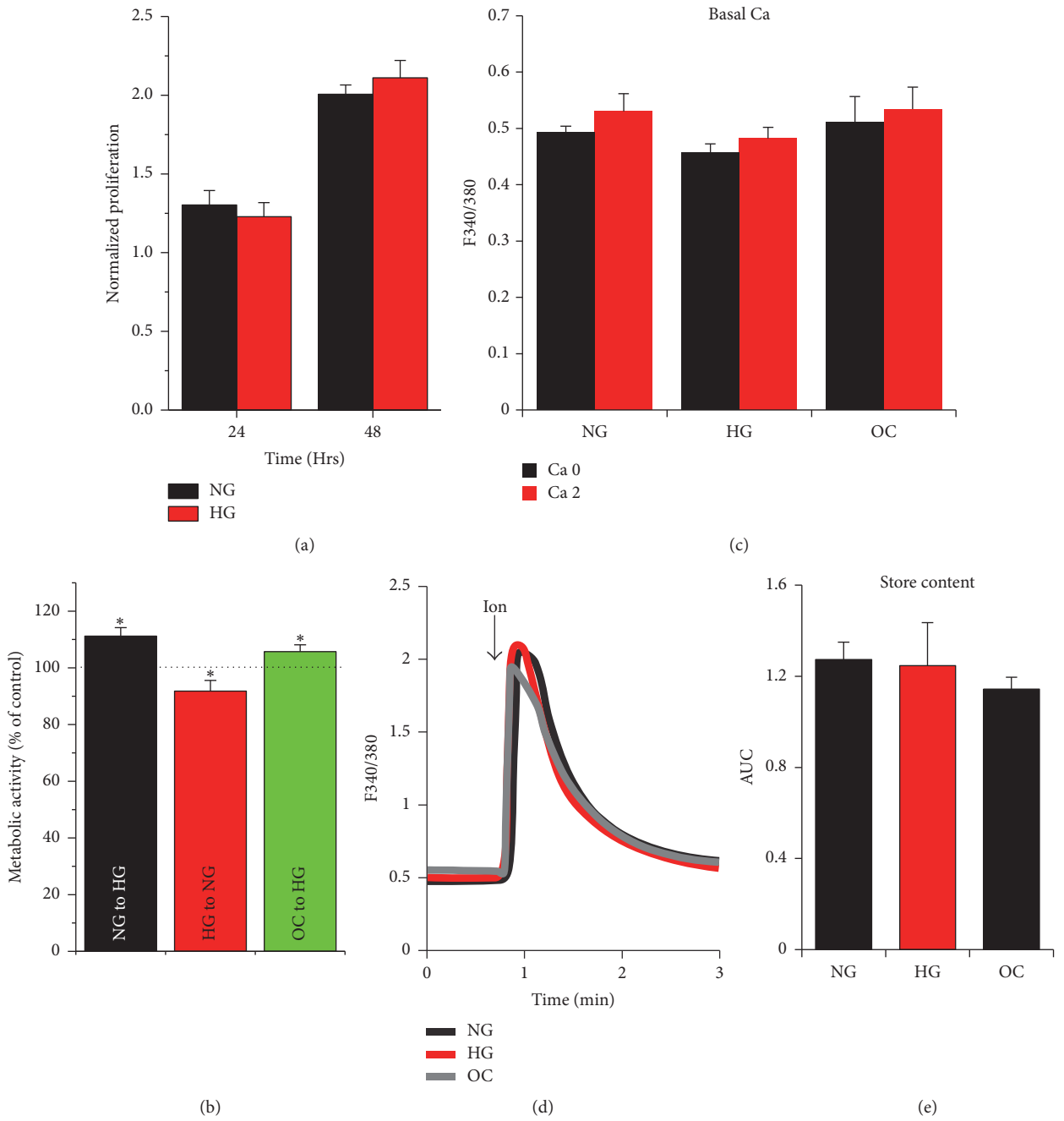

Figure 1: Effect of glucose on proliferation, basal Ca^{2+}, and store Ca^{2+} content in A7r5 VSM cells. Proliferation (a), metabolic activity (b), basal Ca^{2+} level (c), and Ca^{2+} store content (d-e) were tested. Proliferation of A7r5 cells cultured under HG and NG was determined, 24 and 48 h after plating, using trypan blue dye exclusion counting (a). Metabolic activity of A7r5 cells from the different groups (HG, NG, and OC) shifted to HG or NG, measured 3 h later using the WST-1 assay (b). For basal Ca^{2+} levels and store content, cells cultured under HG, NG, or OC were loaded with Fura2-AM (2 μM for 30 min) and the extent of Ca^{2+} release was determined following treatment of the cells with 2 μM ionomycin (d-e). Data are presented as mean ± SE from at least three independent experiments done each in triplicate. $^{*}P < 0.05$.

Ca^{2+} channel, is important for VSM contractility and for regulating the myogenic response in resistance arteries [45–49]. Therefore, modulation of VGCC' activity could impact VSM contractility and function. In order to test the effects of extracellular glucose on VGCC we measured Ca^{2+} influx following a depolarization stimulus with KCl (Figure 4(a)). We could not observe any changes in the Ca^{2+} influx through VGCC, evaluated by measuring the peak amplitudes of KCl-induced Ca^{2+} transient (Figures 4(a) and 4(b)). As expected, the NaCl osmotic control did not stimulate Ca^{2+} influx as compared to KCl depolarization (Figure 4(b), black bars). To investigate whether glucose levels affect VSM contractility in response to Ca^{2+} influx through VGCC, we measured the contractility of A7r5 cells in response to KCl-induced

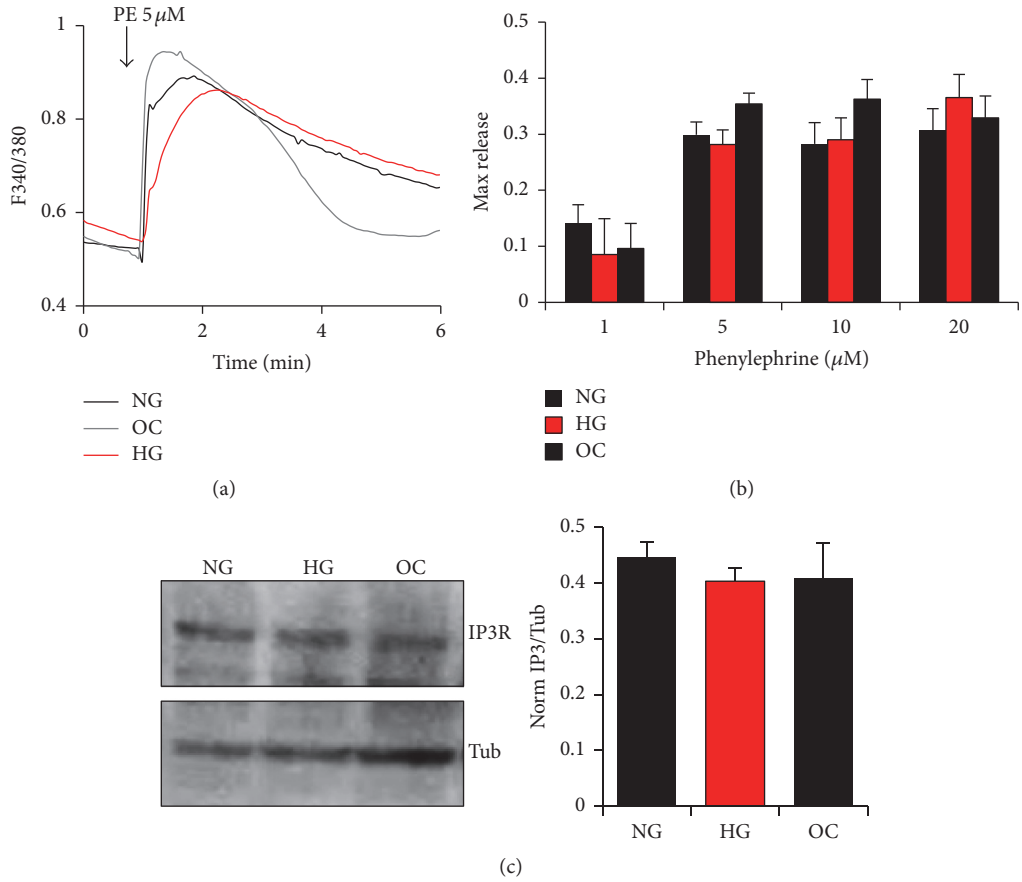

FIGURE 2: Effect of glucose normalization on Ca^{2+} release in A7r5 VSM cells. PE induced Ca^{2+} release (a, b) and IP3R expression (c) in response to different glycemic conditions. Cells cultured under HG, NG, or OC were loaded with Fura-2AM and Ca^{2+} transients imaged following exposure to different concentrations of PE (1–20 μM) (a, b). IP3R expression from cells cultured under HG, NG, and OC was determined by western blot analysis and quantified, as described in Materials and Methods (c). Data are presented as mean ± SE from at least three independent experiments done each in triplicate.

depolarization (Figure 4(c)). As A7r5 cells are adherent, it is difficult to use cell shortening as a measure of contractility. However, contractile fibers, formed in cells stimulated with an agonist or with any Ca^{2+} mobilizing agent, are readily visible under light microscopy. Consequently, we used the previously described imaging approach that enables relative quantification of contractility [50]. Depolarization-induced contraction was similar among the three different groups (Figures 4(c) and 4(d)). These data are in agreement with the conclusion that glucose levels do not modulate the activity of VGCC or the resultant VSM contractions.

3.4. Ca^{2+} Leak from the Endoplasmic Reticulum (ER). Thapsigargin (TG) inhibition of the SERCA pump on the ER membrane demonstrates the presence of a passive leak pathway from the ER. The molecular mechanisms controlling this ER leak pathway remain controversial with roles proposed for the IP_3Rs, presenilins, and the translocon machinery at the ER membrane as potential pathways [51–53]. In order to test whether the ER Ca^{2+} leak is altered in A7r5 cells cultured in HG, the maximum values and the time to half-max for the Ca^{2+} transient induced following treating cells with TG were measured in Ca^{2+}-free Ringer buffer as illustrated in Figure 5(a). Under all three conditions NG, HG, and OC, the peak of the TG-induced Ca^{2+} transient was similar (Figure 5(b)), which is consistent with the ionomycin data (Figure 1(e)). These data show that Ca^{2+} store content is similar under all three conditions. In contrast, the time to reach half-max is significantly slower ($p = 0.023$) in cells grown under HG as compared to NG and OC (Figure 5(c)). Because the rise of the Ca^{2+} transient is partially dependent on Ca^{2+} leak from the ER, this argues that ER Ca^{2+} leak is inhibited when cells are cultured in HG.

3.5. Ca^{2+} Extrusion: Plasma Membrane Ca^{2+}-ATPase (PMCA) and the Na^{+}-Ca^{2+}-Exchanger (NCX). The plasma membrane Ca^{2+}-ATPase (PMCA) and the sodium/calcium exchanger (NCX) are the primary mechanisms for Ca^{2+} extrusion out of the cell to maintain cytosolic Ca^{2+} homeostasis. To test whether Ca^{2+} extrusion is altered when cells are cultured under hyperglycemia condition, we used TG-dependent Ca^{2+} release in Ca^{2+}-free conditions and calculated the rate of decay of the Ca^{2+} transient as a measure of Ca^{2+} extrusion out of the cell (Figure 5(a)). Under these conditions, Ca^{2+} influx is absent in Ca^{2+}-free solution and Ca^{2+} reuptake into

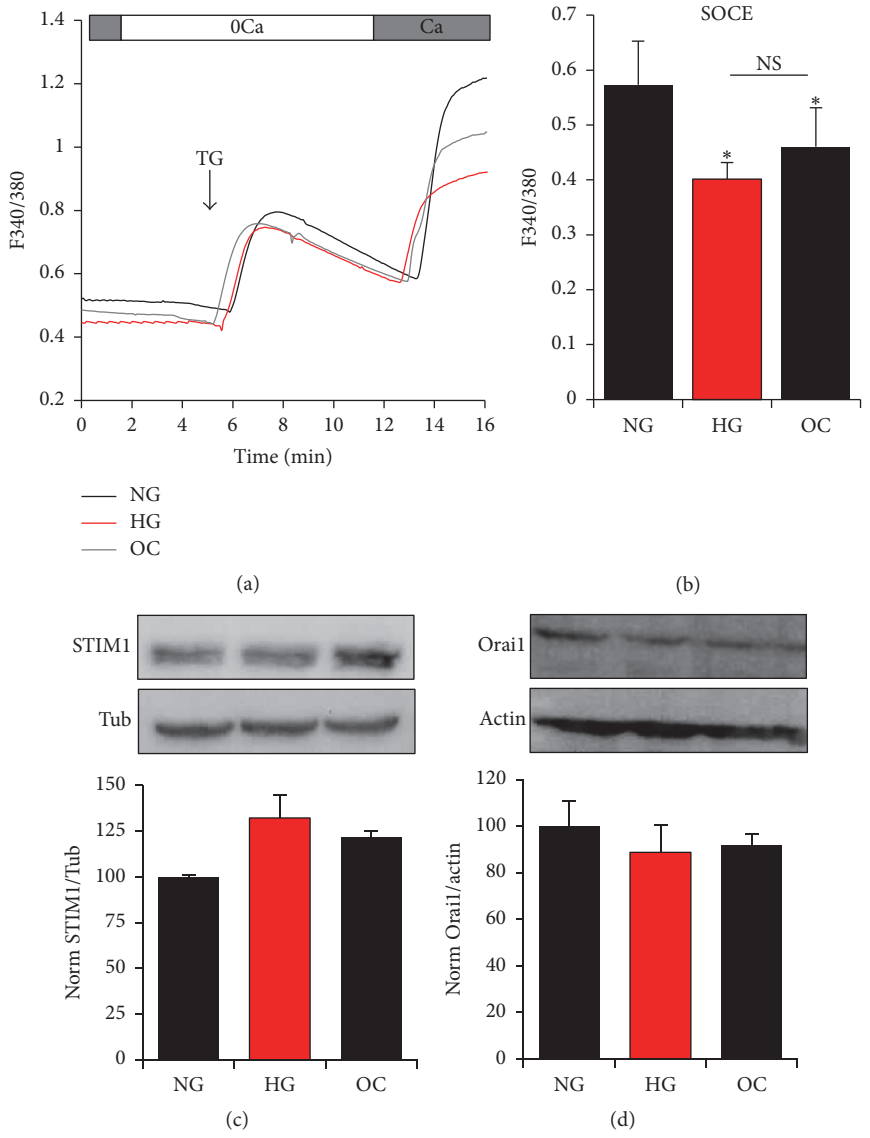

Figure 3: Effect of glucose Ca^{2+} influx in A7r5 VSM cells. Effect of HG and NG on store-operated Ca^{2+} entry (SOCE) (a, b) and on protein expression of STIM and Orai1 (c-d). Cells were loaded with Fura2-AM and SOCE stimulated after store depletion with 1 μM thapsigargin (TG), an irreversible inhibitor of SERCA (a, b). Protein expression of STIM and Orai1 was determined between the different groups by western blot (c-d). Densitometry analysis was performed using Gene Tools, Geliance 600 Imaging system. Data are presented as mean ± SE from at least three independent experiments done each in triplicate. $^*p < 0.05$; NS: not significant.

the ER is blocked by SERCA. Therefore, the only mechanisms that can lower cytoplasmic Ca^{2+} levels are the Ca^{2+} extrusion pathways. As such, the rate of decay of the Ca^{2+} transient under this experimental paradigm offers a measure of Ca^{2+} extrusion. To further differentiate between PMCA and NCX we performed these experiments under normal extracellular Na^+ concentrations or with Na^+ replaced with NMDG to inhibit the activity of NCX (Figures 5(d) and 5(e)). NCX uses the Na^+ gradient across the cell membrane to extrude Ca^{2+} against its concentration gradient; therefore, in the absence of extracellular Na^+, NCX activity is blocked. NCX in the absence of extracellular Na^+ could run in reverse mode, thus transporting Ca^{2+} into the cell. However, this is unlikely to contribute under these experimental conditions since cells are incubated in Ca^{2+}-free solutions.

The rate of Ca^{2+} extrusion in Na^+-containing or NMDG solutions was similar for each glucose treatment (NG, HG) and for the osmotic control (OC), arguing that the primary

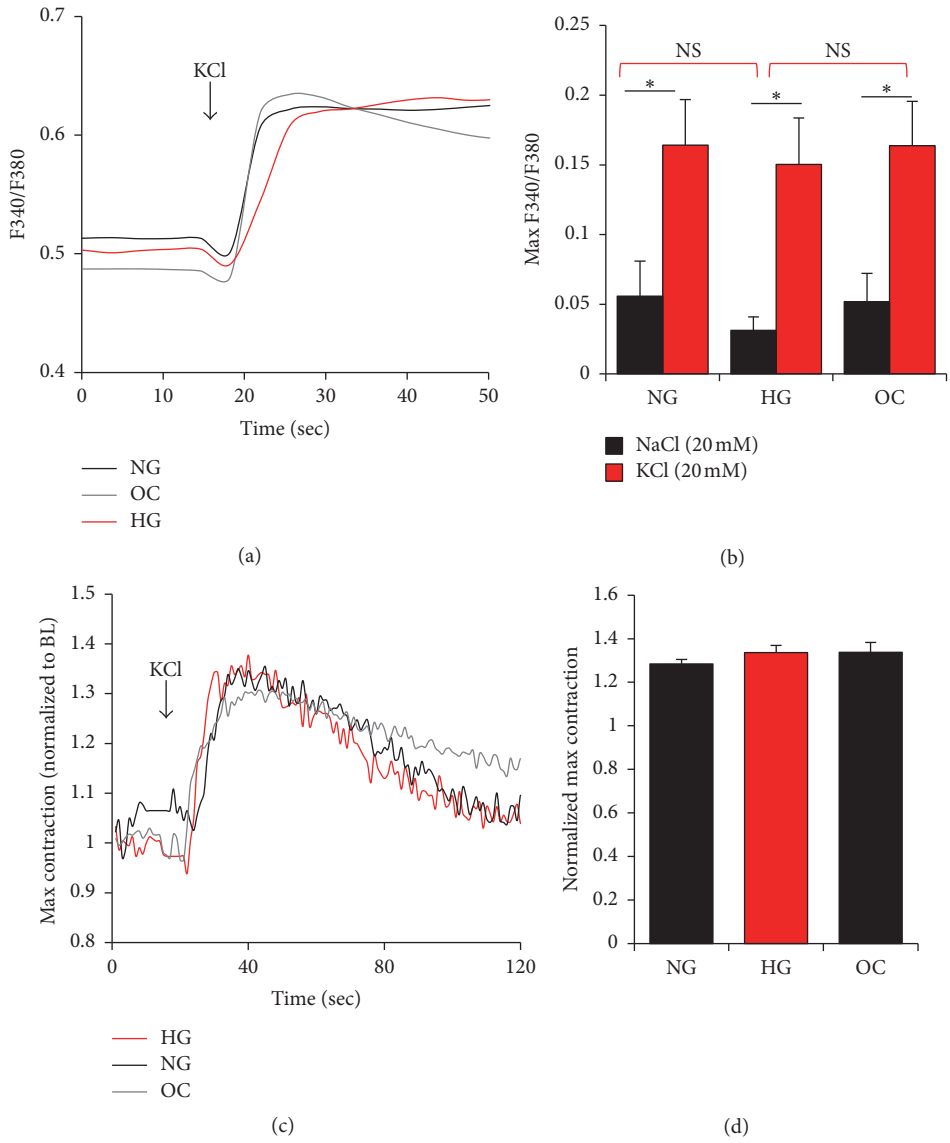

Figure 4: Effect of glucose on voltage gated channels (VGCC) in A7r5 VSM cells. Ca^{2+} transients (a, b) and VSM contraction (c, d) in response to a 20 mM KCl depolarizing pulse to activate VGCC in A7r5 cells. Cytosolic Ca^{2+} transients in Fura2-AM loaded A7r5 cells, cultured under HG, NG, and OC in response to 20 mM KCl (a, b). Effect of glucose normalization on A7r5 cells contractility in response to 20 mM KCl was determined as described in Materials and Methods. Max contraction is presented after normalization to basal level (c, d). Data are presented as mean ± SE from at least three independent experiments done each in triplicate. $^{*}p < 0.05$; NS: not significant.

Ca^{2+} extrusion pathway in A7r5 cells is PMCA (Figure 5(e)). However, incubating cells in HG for prolonged periods of time leads to a small but significant increase in PMCA activity ($p = 0.027$), revealed as a faster decay to half-max ($p = 0.033$) (Figure 5(e)). Consistent with the functional data, NCX protein expression was not statistically different between the different groups (Figures 6(a) and 6(b)), supporting the conclusion that the differential activity observed is due to enhanced PMCA activity. To determine if this increase is due to an increase in PMCA mRNA or protein expression, we focused on the two PMCA isoforms known to be expressed in VSM cells, PMCA1 and PMCA4 [54]. Western blot analyses show an increase in total PMCA protein levels in the HG cultures, that is due to PMCA4 ($p = 0.04$) but not PMCA1 increased protein expression levels (Figures 6(c) and 6(d); Supplemental Figure 2). In contrast, quantitative RT-PCR show no change in PMCA1 and PMCA4 mRNA levels between the different groups (Figure 6(e)).

3.6. Sarcoplasmic Reticulum Ca^{2+}-ATPase (SERCA). To test for changes in the activity of the SERCA pump, which is responsible for Ca^{2+} store refilling after a Ca^{2+} transient, we released Ca^{2+} from intracellular store using phenylephrine and then at the peak of the Ca^{2+} transient a high concentration of lanthanum (La^{3+}, 1 mM) was added to block both Ca^{2+} influx from the extracellular space and Ca^{2+} extrusion pathways [55]. Under these conditions where Ca^{2+} extrusion is blocked the decay kinetics of the Ca^{2+} signal back to

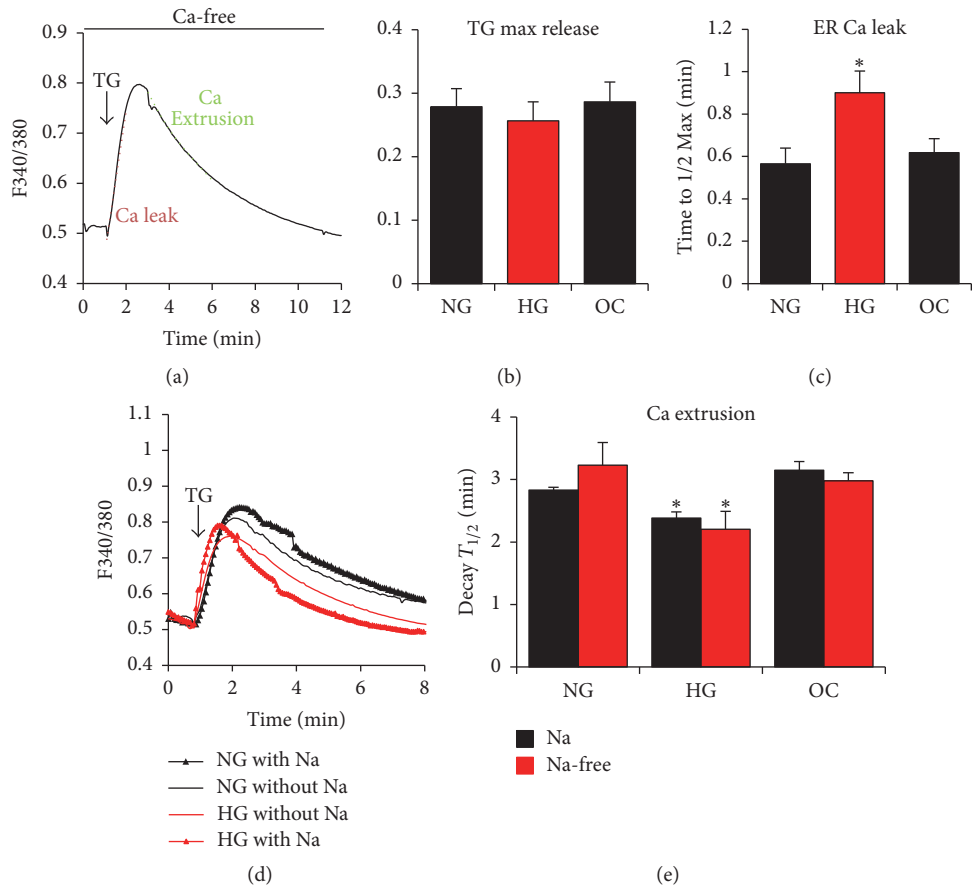

Figure 5: Effect of glucose on Ca^{2+} leak and Ca^{2+} extrusion in A7r5 VSM cells. A7r5 cells, cultured under HG, NG, and OC, were loaded with Fura2-AM and passive leak was monitored after store depletion with 1 μM thapsigargin (TG), an irreversible SERCA inhibitor (a). Peak amplitude in response to TG (TG max release) and the time to maximum release are shown (b, c). Ca^{2+} extrusion, which is presumed to be due to the combined activity of PMCA and NCX, was monitored as the decay of the TG-induced Ca^{2+} transient in Ca^{2+} free conditions to avoid Ca^{2+} influx. To inhibit NCX, sodium was replaced with equimolar concentration of N-methyl-D-glucamine (d-e). Time to half ($T_{1/2}$) decay was then measured and compared between the different conditions (d-e). Data are presented as mean ± SE from at least three independent experiments done each in triplicate. $^{*}p < 0.05$.

baseline reflect SERCA activity (Figure 7(a)). Interestingly, the half-time of decay of the Ca^{2+} signal is significantly longer in cells incubated in HG conditions as compared to NG or OC conditions ($p = 0.001$) (Figures 7(a) and 7(b)). This shows that SERCA activity is inhibited when cells are cultured in HG conditions for extended time periods. Inhibition of SERCA activity is coupled to a small decrease in SERCA2 protein expression levels in HG culture conditions (Figures 7(c) and 7(d)). However, no changes in SERCA2 mRNA levels were detected (Figure 7(e)).

Several lines of evidence support the idea that hyperglycemia induces its damaging effect on SERCA through ROS generation. To validate this hypothesis in our system, A7r5 cells were cultured in HG, NG, or OC in the presence or absence of N-acetyl-cysteine (NAC), a universal radical scavenger, for 2 weeks. Figure 7(f) shows that NAC significantly ($^{**}p = 0.001$) restores HG-induced SERCA compromised activity while no effect was seen on cells grown in NG or OC. These data confirm that oxidative stress is responsible for the decrease in SERCA activity of cells grown under HG. To further confirm the involvement of HG in ROS generation, intracellular ROS levels were determined using the ROS-sensitive fluorescence-generating probe DCF-DA assay. This assay shows that 30 min exposure to HG of cells already grown in NG or OC is sufficient to significantly ($^{*}p < 0.05$) increase ROS generation (Figure 7(g)).

3.7. Human Diabetic VSM Cells. To test whether the changes observed in the A7r5 model of cells cultured under hyperglycemic conditions apply to human VSM cells under diabetic conditions, we obtained primary aortic VSM cells from normal (NHVSMC) and diabetic (Type 2) individuals (DHVSMC) (Lonza, Walkersville, MD). Cells from normal and diabetic individuals were cultured in media containing NG levels (5.5 mM) supplemented with FBS and all necessary cytokines and growth factor as recommended by the supplier. Each batch of cells (normal and diabetic) was used within two weeks of culture. Cells were cultured in normal glucose levels to focus specifically on changes to the Ca^{2+} signaling machinery due to persistent irreversible damage as a result

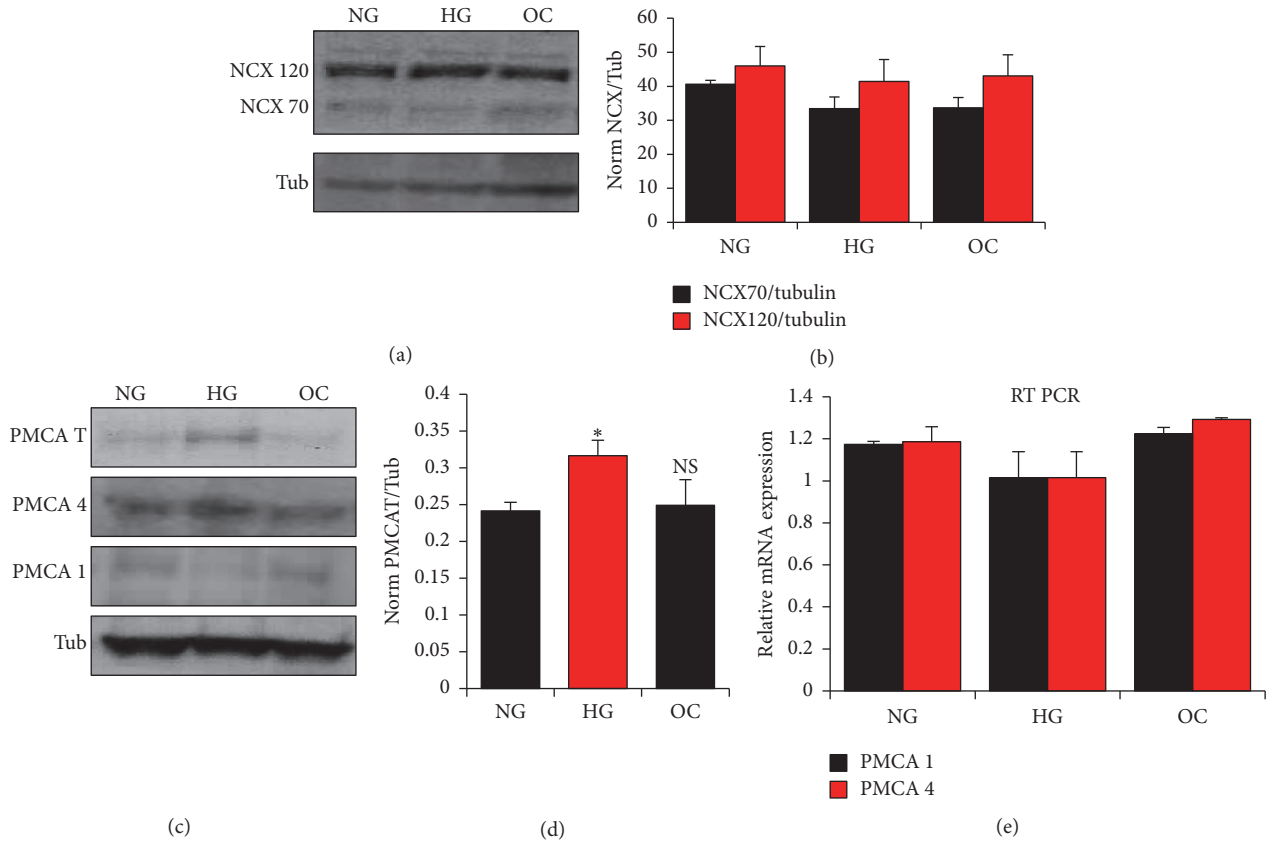

Figure 6: Effect of glucose on PMCA and NCX expression in A7r5 VSM cells. The protein expression of PMCA (total, isoform 1, and isoform 4) and NCX (full length: NCX 120 and proteolytic fragment: NCX 70) were compared in A7r5 cells cultured under HG, NG and OC by western blot analysis (a, c). Densitometry analysis was performed using Gene Tools, Geliance 600 Imaging system (b, d). Gene expression of PMCA 1 and 4 was monitored by quantitative RT-PCR (e). Data are presented as mean ± SE from at least three independent experiments done each in triplicate. $^{*}p < 0.05$; NS: not significant.

of diabetes and not to the culturing conditions. We used this approach based on the findings from the A7r5 model where alterations in Ca^{2+} signaling were observed only after prolonged exposure to hyperglycemia, strongly arguing that they are due to long lasting modifications.

We applied the same approaches described above for A7r5 cells to test for changes in the Ca^{2+} signaling machinery in VSM cells from normal and diabetic individuals. No changes in proliferation rate (Figure 8(a)), basal Ca^{2+} levels (Figure 8(b)), or VSM contraction in response to a KCl depolarization (Figure 8(e)) were observed between cells from normal and diabetic individuals. In contrast, a significant increase ($p = 0.009$) in Ca^{2+} store content, based on the ionomycin releasable pool method, was detected in cells from diabetic individuals as compared to the normoglycemic control (Figure 8(c)). This increased store content was associated with a significant decrease ($p = 0.044$) in SOCE in diabetic VSM cells (Figure 8(d)). A similar decrease in SOCE was observed in A7r5 cells after prolonged exposure to high glucose concentrations, although this seems to be mostly attributable to an osmotic effect (Figure 3(b)).

Using the thapsigargin approach to assess ER Ca^{2+} leak, no changes in the maximal levels of Ca^{2+} release were detected (Figure 9(a)). This is in contrast to the enhanced ionomycin releasable Ca^{2+} pool in diabetic VSM cells (Figure 8(c)). This could be due to slow Ca^{2+} release from the ER following TG treatment, which is dependent on the rate of the passive ER Ca^{2+} leak. In contrast, ionomycin induces a rapid release from stores given its ionophore properties. The slow Ca^{2+} release in response to TG allows time for other pathways such as Ca^{2+} extrusion out of cell to act on the released Ca^{2+}, which would mask the real store Ca^{2+} content.

Other aspects of Ca^{2+} signaling, including the response to the G-protein coupled agonist PE (Figure 9(c)), Ca^{2+} extrusion (Figure 9(d)), and importantly SERCA activity (Figure 9(e)), were not statistically different among the normal and diabetic VSM cells. Although SERCA activity showed a trend toward inhibition in diabetic human VSM cells, this did not reach statistical significance (Figure 9(e)). This is in contrast to the dramatic inhibition of SERCA activity observed in A7r5 cells exposed to high glucose for prolonged time periods (Figure 7(b)), which was the most pronounced alteration to Ca^{2+} signaling observed in this model.

To assess the expression levels of the different Ca^{2+} channels and transporters in human VSM cells, we used real-time

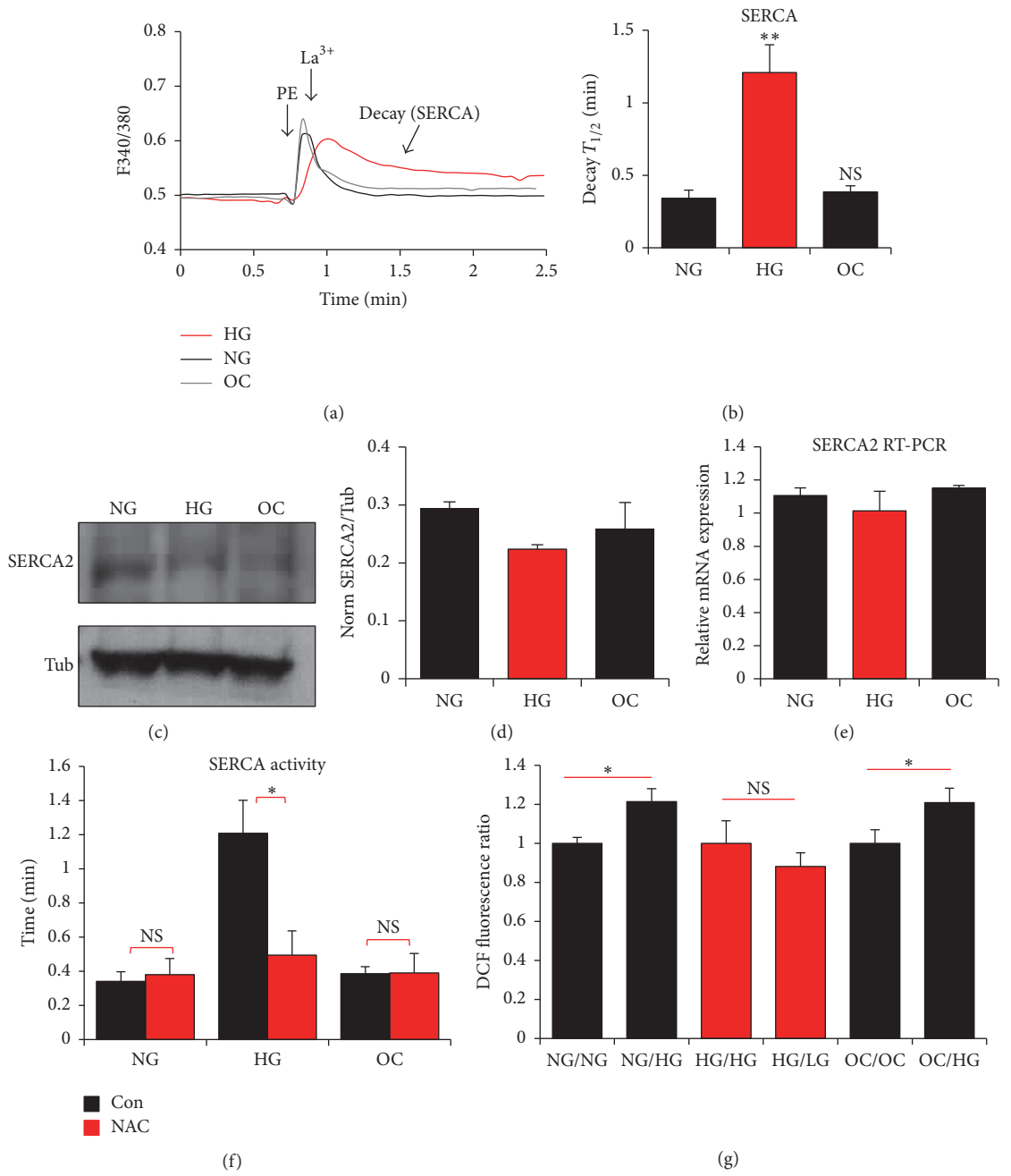

Figure 7: Effect of glucose on sarcoplasmic reticulum ATPase (SERCA) activity in A7r5 VSM cells. A7r5 cells, cultured under HG, NG, and OC, were loaded with Fura2-AM and cytosolic Ca^{2+} was monitored following treatment with 20 μM PE and 1 mM lanthanum chloride (La^{3+}) at the time points indicated by the respective arrows (a). Time to half decay after the La^{3+} treatment was then measured and compared between the different conditions (b). Protein and gene expression of SERCA from A7r5 cells cultured under the different condition were determined by western blot and real-time PCR, respectively (c–e). Effect of NAC on HG-induced SERCA compromised activity was determined in A7r5 cells cultured in the presence of NAC for 2 weeks prior to the determination of SERCA activity (f). The effect of HG on ROS generation in cells shifted for 30 min from NG and OC to HG and from HG to NG (g) was measured by the DCFH assay. Data are presented as mean ± SE from at least three independent experiments done each in triplicate. $^{**}P < 0.005$.

PCR to evaluate mRNA levels of PMCA1, PMCA4, Orai1, Orai2, stim1, STIM2, and SERCA1-3. No significant changes were detected in any of the studied markers (Supplemental Figure 3).

4. Discussion

Ca^{2+} signaling is an integral signaling module involved in many aspects of cellular physiology. Specificity of Ca^{2+}

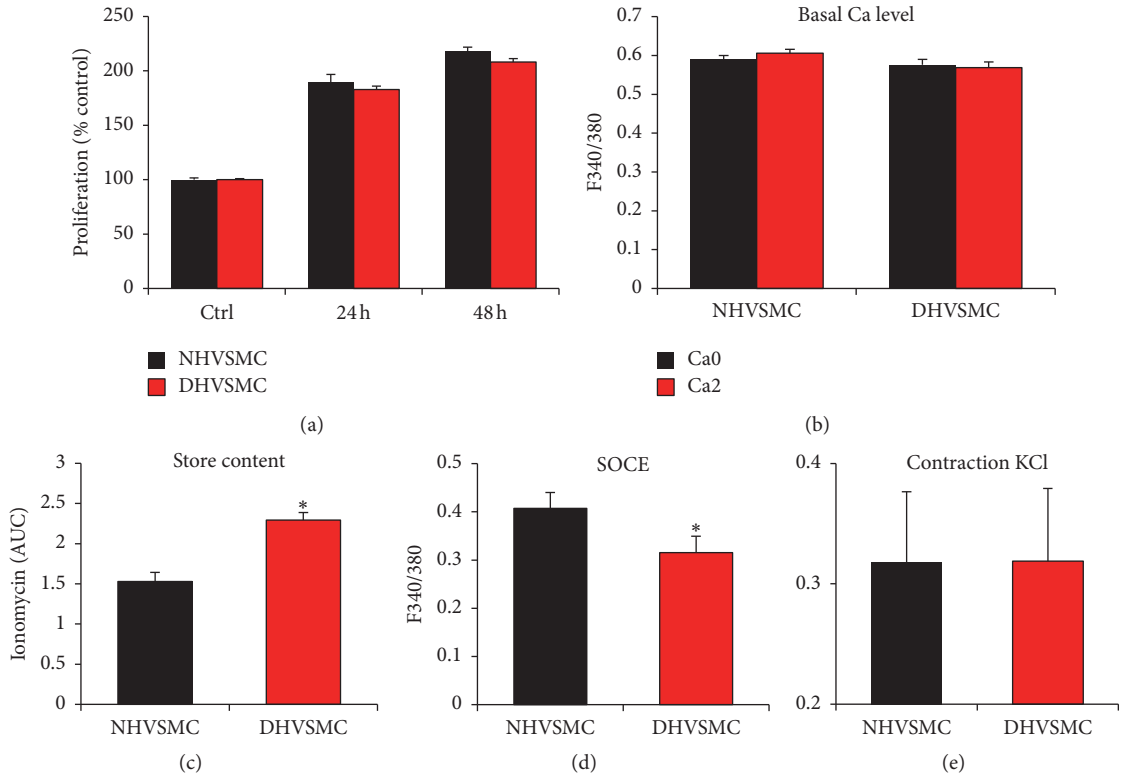

FIGURE 8: Proliferation and Ca^{2+} signaling in VSMC cells from normal (NHVSMC) and diabetic (DHVSMC) human aorta. For all the tests human normal and diabetic VSM cells were cultured under NG as recommended by the supplier. Cell proliferation was determined 24 and 48 h after plating, using the WST-1 assay (a). For Ca^{2+} basal levels and Ca^{2+} store content, cells were loaded with 2 μM Fura-2AM for 30 min and the extent of Ca^{2+} release was determined following treatment of the cells with 2 μM ionomycin (b-c). For SOCE measurement, cells were loaded with 2 μM Fura-2AM for 30 min and SOCE was determined after store depletion with 1 μM thapsigargin (TG) (d). Cell contractility in response to 20 mM KCl was determined as described in Materials and Methods (e). Data are presented as mean ± SE from at least three independent experiments done each in triplicate. $^{*}p < 0.05$.

signaling stems from the integrated contribution of different channels and transporters that move Ca^{2+} across disparate subcellular compartments leading to complex spatial and temporal dynamics to encode specific downstream cellular functions [56]. As such Ca^{2+} signals are able to encode various cellular responses based on the specific dynamics of Ca^{2+} transients. As such, alterations in the function or regulation of Ca^{2+} transporting proteins could have significant consequences on cellular and organismal physiology [56–58].

Several reports show that vascular dysfunction in the context of hyperglycemia is associated with alterations to multiple signaling pathways, including advanced glycation end-products (AGEs), PKC-DAG, and the hexosamine pathways [16, 24–28]. These pathways could directly or indirectly affect Ca^{2+} signaling, which in turn would contribute to vascular dysfunction (VD) by altering VSM or endothelial cell physiology. Interestingly, a recurring pathway that appears to be involved in the aforementioned mechanisms is the generation of reactive oxygen species (ROS) [24–28]. Furthermore, several lines of evidence suggest that oxidative stress caused by hyperglycemia increases the risk of the adverse cardiovascular events associated with diabetes [10, 11, 21, 26, 59]. For instance, in comparison to normal cells, an increase in ROS generation has been reported in smooth muscles from diabetic human uterine vessel [26]. It has been further shown that chronic treatment with antioxidants normalizes VD associated with diabetes supporting the deleterious role of ROS on vascular function [26, 59].

Our data show that short-term (up to 72 hrs) exposure of A7r5 vascular smooth muscle cells to hyperglycemic conditions does not result in any detectable changes in Ca^{2+} signaling. However, long-term culture of A7r5 cells in HG versus NG conditions leads to significant changes to Ca^{2+} signaling. Most significantly long-term exposure to hyperglycemic conditions is associated with an inhibition of both Ca^{2+} leak from the ER (Figure 5(c)) and SERCA-dependent Ca^{2+} reuptake into the ER (Figure 7(b)). The fact that these changes required culture under hyperglycemic conditions of more than 4 weeks argues that they are not driven by metabolic changes due to the increased glucose load, but rather that they are associated with modifications such as glycation or other cellular adaptations to the hyperglycemic conditions. Consistent with this conclusion, Fleischhacker et al. found no differential effect in the response of diabetic or normal smooth muscle cells to KCl and norepinephrine after they were isolated and cultured for 4 weeks [26].

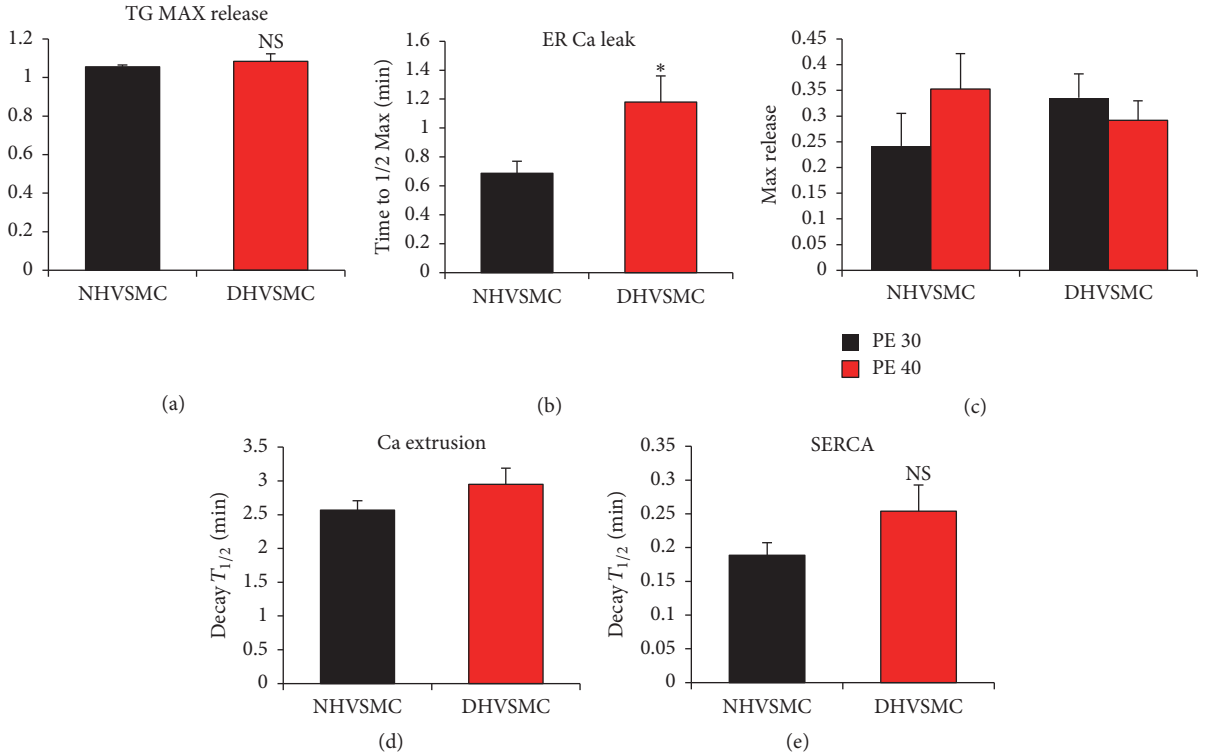

Figure 9: Ca^{2+} signaling pathways in normal (NHVSMC) and diabetic (DHVSMC) human aortic VSM cells. Cells were loaded with 2 μM Fura-2AM for 30 min and passive leak was monitored after store depletion with 1 μM thapsigargin (TG). Peak amplitude in response to TG (TG max release) and the time to half maximum release were compared between normal and diabetic cells (a, b). For the extrusion pathways the activity of PMCA and NCX was monitored by Ca^{2+} imaging after store depletion with 1 μM TG. Time to half ($T_{1/2}$) decay was then measured and compared between the different conditions (c). Cells were labeled with 2 μM Fura-2AM for 30 min and Ca^{2+} release was imaged following treatment of the cells with 30 and 40 μM of PE (d) or by using 30 μM PE followed by 1 mM lanthanum chloride (La^{3+}) (e). Time to half decay was then measured and compared between normal and diabetics cells (e). Data are presented as mean ± SE from at least three independent experiments done each in triplicate. $^{*}p < 0.05$; NS: not significant.

Nevertheless, significant differences were observed when the cells were freshly isolated arguing that they are due to the pathophysiological state of the diabetic organism, potentially due to circulating factors, rather than irreversible changes at the cellular level. These effects were reversed following treatment with superoxide dismutase and when glycated products were removed. This argues that observed differential responses are not due to genetic modifications but require the presence of glycated products.

Furthermore, the contribution of HG-induced ROS to the observed SERCA inhibition has been previously reported [10]. Tong et al. showed that ROS-induced oxidation of Cys647 of SERCA inhibits nitric oxide- (NO-) induced-S-glutathionylation of SERCA leading to a decrease in its activity [10]. Therefore, to confirm that long-term normalization of glucose rescues SERCA activity by decreasing oxidative stress, we cultured A7r5 cells for 2 weeks in the presence of N-acetyl-cysteine (NAC), a strong radical scavenger. NAC normalized SERCA activity (Figure 7(f)). The involvement of ROS was further confirmed by the use of the DCFH assay that showed that exposure of cells already cultured in NG or OC media to HG for 30 min was sufficient to increase ROS levels (Figure 7(g)).

Culturing VSM cells under hyperglycemic conditions is an easy experimental approach to discern the effects of hyperglycemia on VSM function and has been used by several groups. The conclusion from our experiments and those of others are that there are long-term alterations that are associated with changes to VSM physiology exposed to hyperglycemia. We were as such interested in testing whether changes to Ca^{2+} signaling observed in A7r5 cells exposed for prolonged periods of time to hyperglycemia are replicated in primary VSM cells from human subjects with diabetes.

In diabetic human VSM cells, we observe an increase in ER Ca^{2+} store content (Figure 8(c)), inhibition of SOCE (Figure 8(d)), and an inhibition of passive Ca^{2+} lead from the ER ($p = 0.02$) (Figure 9(b)). Surprisingly, the most dramatic change to the Ca^{2+} signaling machinery observed in A7r5 cells, which is the inhibition of SERCA activity, was not statistically significant in human diabetic VSM cells (Figure 9(e)). This leaves the inhibition of ER Ca^{2+} leak as the only Ca^{2+} signaling pathway tested that consistently shows inhibition in both diabetic human VSM cells and the A7r5 cell culture model.

SOCE is inhibited in both the A7r5 model and human diabetic VSM cells; however, the fact that a similar inhibition

was observed in the osmotic control in the A7r5 model in our hands casts doubt as to whether this inhibition is due to hyperglycemia per se.

There is compelling evidence from animal and clinical studies that some of the adverse effects of hyperglycemia on VD associated with diabetes are everlasting and cannot be reversed when glucose levels are improved or controlled [25]. In the context of Ca^{2+} signaling, we show here that the passive Ca^{2+} leak from the ER is consistently inhibited in VSM cells exposed to prolonged hyperglycemia both in humans and in culture model. In human diabetic VSM cells, consistently, this is associated with higher Ca^{2+} store content. However, these changes in Ca^{2+} dynamics were not associated with alteration to VSM Ca^{2+} signals in response to PE or to basal cytoplasmic Ca^{2+} levels. Therefore, their physiological significance remains unclear. Nonetheless, one could envision agonist stimulation in vivo leading to a more pronounced Ca^{2+} transient given the increased Ca^{2+} store content, which could result in a more sustained Ca^{2+} signal, thus affecting VSM physiology and contractility.

Hyperglycemia has also been shown to affect the microvasculature by impacting the activation state of the calcineurin-NFAT signaling cascade in smooth muscle cells of resistance arteries [60], a process that seems to be related to upregulation of SOCE in the endothelium in response to high glucose levels [37]. These changes could affect vascular health and contractility, thus leading to complications. There is indeed evidence supporting a decreased responsiveness of small mesenteric arteries to phenylephrine in diabetic as compared to control mice [61]. Therefore, hyperglycemia affects the responsiveness of microvessels and their contractility, which would ultimately contribute to the vascular dysfunction that is tightly associated with diabetes. Hence, understanding the molecular mechanisms through which hyperglycemia affects the function of both endothelial and VSM cells and their interactions, particularly in small resistance arteries, becomes critical.

Finally, an important take-home message from our experiments is that changes observed in a cell culture model of hyperglycemia need to be interpreted with caution as they are not readily translatable to VSM cells from diabetic subjects. This is not surprising given that the type and extent of exposure of VSM to hyperglycemia in the whole organism tend to be intermittent and it occurs in the context of a multitude of additional factors acting on VSM cells.

Disclosure

Nahed El-Najjar current address is as follows: University Hospital Regensburg, Institute of Clinical Chemistry and Laboratory Medicine, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany. The statements made herein are solely the responsibility of the authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Rashmi P. Kulkarni and Nancy Nader have equal contribution.

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