CARDIOVASCULAR DISEASE AND DIABETES: A JOURNEY FROM BENCH TO BEDSIDE

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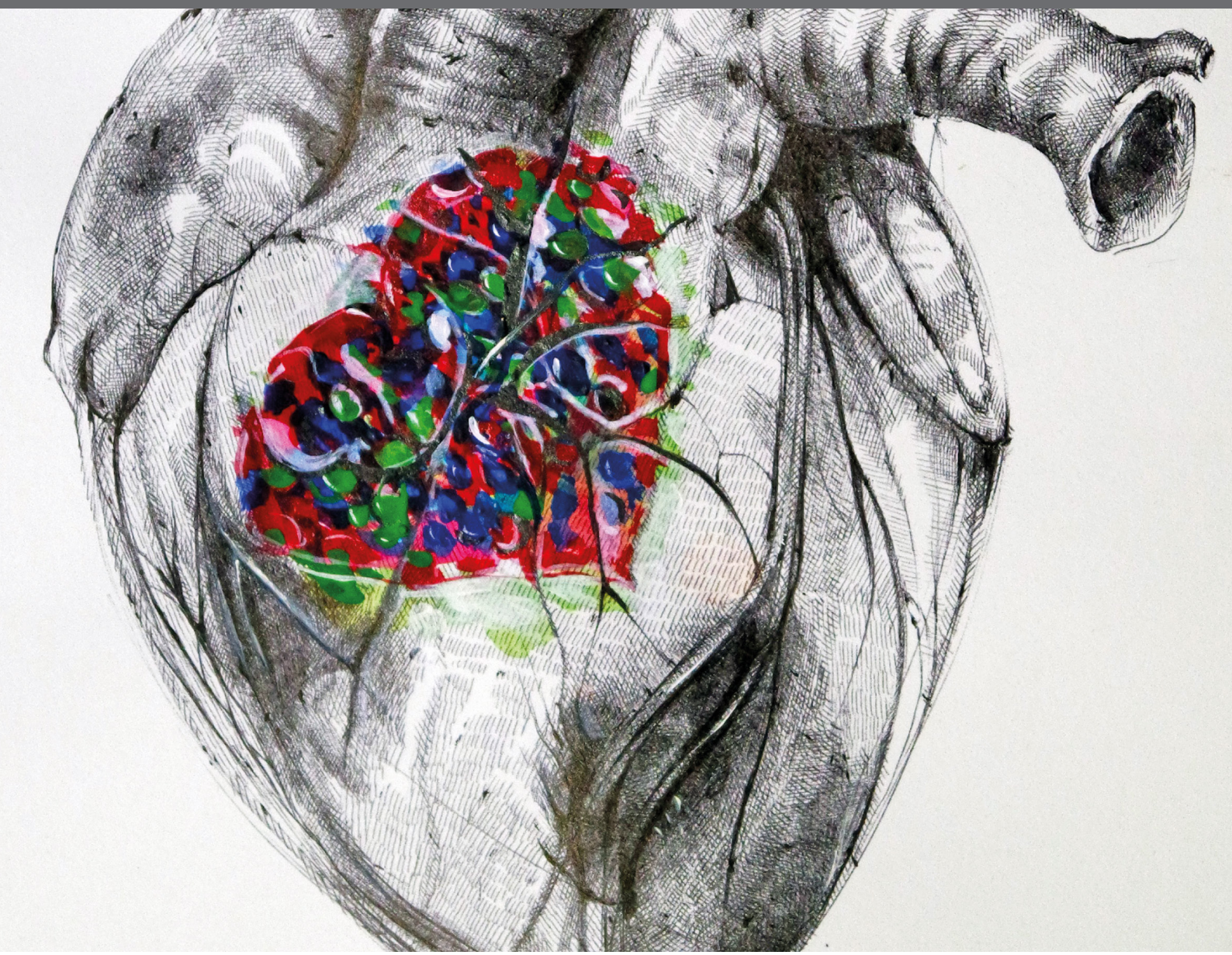

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CARDIOVASCULAR DISEASE AND DIABETES: A JOURNEY FROM BENCH TO BEDSIDE

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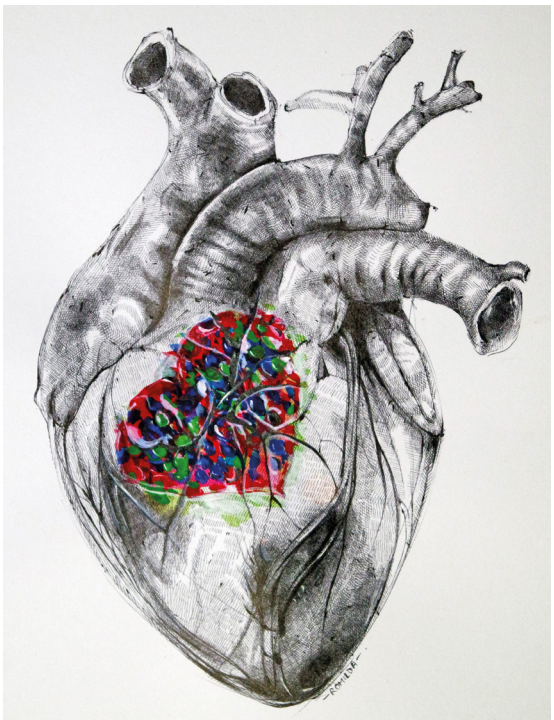

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People with diabetes mellitus have a higher-than-average risk of having a heart attack or stroke. However, the molecular mechanisms underlying the relationship between diabetes and cardiovascular disorders are not fully understood; therefore, successful attempts at designing rational interventions remain limited. Nonetheless, recent advances have opened numerous areas of investigation exploring this rapidly evolving research field, also showing the other side of the coin, i.e., how cardiovascular disease can affect insulin release and glucose homeostasis. The present eBook aims to present some of the more relevant and recent acquisitions on the molecular mechanisms linking diabetes and cardiovascular disease, maintaining a focus on the actual translatability in clinical practice.

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EDITORIAL
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Editorial: Cardiovascular Disease and Diabetes

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Keywords: diabetes mellitus, endocrinology, microRNA, HFpEF (heart failure with preserved ejection fraction), obesity, metabolic syndrome, EPC — endothelial progenitor cells, human induced pluripotent stem cell (iPSC)

Editorial on the Research Topic

Cardiovascular Disease and Diabetes

People with diabetes mellitus (DM) have a higher-than-average risk of having a heart attack or stroke (1, 2). In fact, DM represents a crucial risk factor for cardiovascular disease (3–5). However, the molecular mechanisms underlying the relationship between DM and cardiovascular disorders are not fully understood; therefore, successful attempts at designing rational interventions remain limited. Nonetheless, recent advances have opened numerous areas of investigation exploring this rapidly evolving research field (6–9), also showing the other side of the coin, i.e., how cardiovascular disease can affect insulin release and glucose homeostasis (10). The present Research Topic aims to present some of the more relevant and recent acquisitions on the molecular mechanisms linking DM and cardiovascular disease, maintaining a focus on the actual translatability in clinical practice.

De Rosa et al., from Magna Graecia University, elegantly illustrated fundamental genetic and epigenetic mechanisms linking cardiovascular disease and DM; similarly, Pordzik et al. identified the functional role of specific platelet-related microRNAs in the pathophysiology of cardiovascular events in high-risk populations, including diabetic patients.

Soares Felicio et al. demonstrated an association between reduced levels of Vitamin D and the presence and severity of diabetic kidney disease in type 1 DM (T1DM); the molecular mechanisms underlying diabetic nephropathy have been also explored by Zou et al. in streptozotocin-induced DM. Arcangeli et al. found a significant association between the number of circulating endothelial progenitor cells (cEPCs) and the age and duration of the disease in T1DM patients: indeed, young T1DM patients have significantly higher levels of cEPCs compared to adult T1DM patients; of note, such difference is also maintained when the disease lasts for more than 10 years. The Authors propose that maintaining a high number of cEPCs, possibly through an efficient glycemic control, would contribute to contain the cardiovascular burden in T1DM. Notably, *in vitro* experiments performed by Lin et al. at New York University have shown how to ameliorate purification and maturation of human induced pluripotent stem cell (iPSC)-derived cardiomyocytes through means of culture in glucose-depleted medium supplemented with fatty acids (oleic acid and linoleic acid) and 3,3′,5-triiodo-L-thyronine (T3).

Applying comprehensive analyses based on imaging and molecular biology, Infante et al. revealed a greater severity of coronary artery disease in type 2 diabetes (T2DM) patients compared to non-diabetic individuals; equally important, van Bussel et al. from Maastricht University Medical Center, highlighted the actual advantages of multiparametric neuroimaging in the clinical evaluation of cognitive decline in T2DM.

The studies performed by Orosz et al. in subjects with impaired glucose tolerance, a prediabetic condition, have shown that prediabetes is associated with repolarization instability, indicated by elevated values of beat-to-beat short-term QT interval variability, thereby suggesting

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that an impaired autonomic control precedes the actual onset of diabetes. Last but not least, Altara et al. validated the key importance of targeting microvascular disease, common in both diabetes and obesity, in order to treat heart failure with preserved ejection fraction (HFpEF). Microvascular disease is a growing public health problem, accounting for approximately half of hospital admissions of individuals with heart failure (1, 5, 11, 12).

In summary, the present Research Topic indicates that the exceptional advances achieved in the last decade in understanding the molecular alterations involved in the pathophysiology of both DM and cardiovascular disease are opening new therapeutic opportunities for the treatment of these disorders and, potentially, their future application to the clinical scenario might result to further enhancements in patient care. Furthermore, the exciting findings discussed herein might foster community awareness of these important diseases and stimulate further research in the field.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Type 2 Diabetes Mellitus and Cardiovascular Disease: Genetic and Epigenetic Links

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Type 2 diabetes mellitus (DM) is a common metabolic disorder predisposing to diabetic cardiomyopathy and atherosclerotic cardiovascular disease (CVD), which could lead to heart failure through a variety of mechanisms, including myocardial infarction and chronic pressure overload. Pathogenetic mechanisms, mainly linked to hyperglycemia and chronic sustained hyperinsulinemia, include changes in metabolic profiles, intracellular signaling pathways, energy production, redox status, increased susceptibility to ischemia, and extracellular matrix remodeling. The close relationship between type 2 DM and CVD has led to the common soil hypothesis, postulating that both conditions share common genetic and environmental factors influencing this association. However, although the common risk factors of both CVD and type 2 DM, such as obesity, insulin resistance, dyslipidemia, inflammation, and thrombophilia, can be identified in the majority of affected patients, less is known about how these factors influence both conditions, so that efforts are still needed for a more comprehensive understanding of this relationship. The genetic, epigenetic, and environmental backgrounds of both type 2 DM and CVD have been more recently studied and updated. However, the underlying pathogenetic mechanisms have seldom been investigated within the broader shared background, but rather studied in the specific context of type 2 DM or CVD, separately. As the precise pathophysiological links between type 2 DM and CVD are not entirely understood and many aspects still require elucidation, an integrated description of the genetic, epigenetic, and environmental influences involved in the concomitant development of both diseases is of paramount importance to shed new light on the interlinks between type 2 DM and CVD. This review addresses the current knowledge of overlapping genetic and epigenetic aspects in type 2 DM and CVD, including microRNAs and long non-coding RNAs, whose abnormal regulation has been implicated in both disease conditions, either etiologically or as cause for their progression. Understanding the links between these disorders may help to drive future research toward an integrated pathophysiological approach and to provide future directions in the field.

Keywords: type 2 diabetes mellitus, cardiovascular disease, genetic polymorphisms, high-mobility group A1 variant, epigenetics

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INTRODUCTION

Type 2 diabetes mellitus (DM) is a complex metabolic disease in which concomitant insulin resistance and beta-cell impairment lead to hyperglycemia, which is the hallmark of the disease (1). Its prevalence is in rapid and progressive rise, due to the increase in average life expectancy, growing prevalence of obesity, and westernization of lifestyles in developing countries (2, 3), while its long-term complications are the major causes of morbidity, mortality, and exceptional healthcare costs (4, 5).

Cardiovascular disease (CVD) represents a leading health problem worldwide (6). Prospective studies have demonstrated that diabetic patients have a two- to fourfold propensity to develop coronary artery disease (CAD) and myocardial infarction (MI) (7), establishing that type 2 DM is an independent risk factor for stroke and heart disease (8). Indeed, about 70% of type 2 DM at an age ≥65 years die from CVD (7), while type 2 DM patients with no history of CAD have an equal cardiovascular risk as patients with previous MI (9). CVD and type 2 DM share several common pathophysiological features that are summarized in **Table 1**. Classical cardiovascular risk factors, such as dyslipidemia, hypertension and obesity can also raise the risk of type 2 DM. In particular, insulin resistance and hyperglycemia are associated with a low-grade inflammation, as well as with chronic enhancement of oxidative stress, triggering endothelial dysfunction and promoting atherogenesis (10–12). Among the different soluble mediators associated with the above-mentioned aspects, IL-1β, IL-6, tumor necrosis factor (TNF)-α, and CRP are worth mentioning (13). In addition, it is well documented that type 2 DM is associated with enhancement of platelet and hemostatic activities (14).

Currently, a number of evidences exists, demonstrating that the interaction of type 2 DM and related cardiovascular risk underpin the progressive nature of the vascular damage, leading to atherosclerosis (23), while it is also proved that lifestyle modifications, such as physical activity and weight loss, counteract CVD risk factors in prediabetic individuals (23, 24). As diabetes shares many risk factors with CVD, while some other ones may be independent, this reinforces the postulate proposed by Stern, according to which both diseases come independently from a "common soil" (20). In this scenario, as type 2 DM and CVD are both complex diseases, common risk factors predisposing to these disorders may include shared genetic factors, a setting that has been only partly elucidated.

Many common single-nucleotide polymorphisms (SNPs) have been already associated with an increased risk of CVD and type 2 DM (25), while their search is still ongoing. In addition, novel links between these disorders come from epigenetic studies. In this review, we will try to address the current knowledge about the genetic links between type 2 DM and CVD, and to evidence their potential pathophysiological role in the context of these diseases. We will dedicate a special focus to the high-mobility group A1 (HMGA1) common variant rs139876191, previously identified by us as a susceptibility locus for type 2 DM (26), and recently also associated with MI (27). In addition, we intend to provide an overview about the epigenetic links between type 2 DM and CVD to widen our understanding about the biological mechanisms that join these disorders. More recently, non-coding RNAs have emerged as key regulators of the pathophysiology underlying both type 2 DM and CVD (28–30), adding up to the fast-growing list of common background in the epigenetic regulation between type 2 DM and CVD. However, these mechanisms are often addressed within a specific pathological context, whereas an integrated approach should be preferred in order to capture all potential interlinks between type 2 DM and CVD.

GENETIC ASPECTS

Monogenic Components

Although the most common forms of type 2 DM and the vast majority of CVD are polygenic, Mendelian forms have also been described for both conditions, in which a single gene mutation can trigger the disease (31, 32). In this regard, heterozygous mutations in candidate genes can be at the basis of familial forms of cardiovascular risk factors, including hypertension, hypercholesterolemia and type 2 DM (32). However, such genes do not automatically predispose to both type 2 DM and CVD. For example, recent studies have described a protective role against type 2 DM of *LDL* receptor or *Apo B* gene mutations, the most commonly studied genes for familial hypercholesterolemia. Being this condition characterized by impaired intracellular transport of cholesterol, this suggests a mechanistic role of cholesterol metabolism in type 2 DM (33).

Genetic Polymorphisms

Loci Associated with Type 2 DM and CVD

Many research reports have addressed genetic variants associated with CVD or type 2 DM (34, 35), and the list of loci joint to each specific disease is progressively increasing, mostly due to the power of genome-wide association studies (GWAS), combined with the analysis of large cohorts of patients. Up to

TABLE 1 | Common pathophysiology of type 2 diabetes mellitus (DM) and cardiovascular disease (CVD).

Status	Description	Reference
Insulin resistance	Insulin resistance is one of the most important antecedent of type 2 DM and CVD	(15)
Inflammation	There is a strong relationship between insulin-resistant states, inflammation, and CVD	(14, 16)
Oxidative stress	Chronic oxidative stress contributes to the pathogenesis of insulin resistance, type 2 DM, and CVD	(17)
Hypercoagulability	Enhanced activation of platelets and coagulation factors is reported in patients with type 2 DM and CVD	(13, 18)
High blood pressure	A positive association exists between hypertension, type 2 DM, and the risk of CVD	(19)
Dyslipidemia	Diabetic dyslipidemia is a major link between DM and the increased cardiovascular risk of diabetic patients	(20, 21)
Obesity	Obesity is a major risk factor for type 2 DM and CVD	(22)

now, at least 83 loci have been associated with type 2 DM (36), and more than 30 with CVD (37). As type 2 DM and CVD are linked by common pathophysiological mechanisms, share many risk factors, and display highly correlated phenotypes, different approaches—including candidate gene studies, linkage analyses, and GWAS—have been employed to search for genes predisposing to both diseases. Current findings are summarized in **Table 2**.

Among candidate genes, several ones involved in pathways pathophysiologically related to both diseases, have been extensively investigated. One of them, *paraoxonase*, synthesizes an enzyme bound to high-density lipoprotein (HDL) particles, with a role in protecting LDL from proatherogenic, oxidative modifications. *Paraoxonase* variants have been described, which lead to reduced enzymatic activity or reduced levels of circulating enzyme, such as the paraoxonase polymorphism Gln-Arg 192, or Met-Leu 54, which are independently associated with both type 2 DM and CVD (48–51). As oxidative stress is a major contributor to atherogenesis in diabetic complications (55), further studies have examined other genes involved in the redox balance. The superoxide dismutase (SOD) 2 is one of the key antioxidant defense systems against free radicals. Ala16Val (rs4880) is the *SOD 2* most commonly described gene variant and resulted in a higher risk to develop CVD in diabetic women (52). Other interesting candidate genes for diabetes and CVD are represented by adiponectin and its pathway. Adiponectin is an adipokine with anti-inflammatory and antiatherogenic effects. Reduced levels of this biomolecule, as in obesity, correlate with increased risk for type 2 DM and CVD, whereas higher levels of adiponectin protect from the risk of CVD in diabetes (56, 57). In patients with type 2 DM, the +276 G/T SNP of the *adiponectin* gene has been reported to be associated with CAD (38). The adiponectin receptor 1 (*ADIPOR1*) gene has been found to be another interesting candidate gene for CVD in diabetic subjects. In particular, common haplotypes tagging three SNPs (rs7539542, rs10920531, and rs4950894) and causing reduced *ADIPOR1* gene expression were found significantly associated with CAD in type 2 DM (39). Furthermore, in type 2 DM, an *ADIPOR1* gene promoter variant (rs266729) has been linked with oxidative stress and cardiovascular risk (40).

One of the most associated spot for MI and CAD, identified by GWA strategies in cohorts of different ethnicities (58, 59), is a 58 Kb non-coding region on chromosome 9p21, localized close to the *CDKN2A* and *CDKN2B* genes, in the context of a known non-coding RNA locus (ANRIL). This same region has turned out to be associated with type 2 DM and several cancers in some studies (60–63). Intriguingly, while the proximity to *CDKN2A* and *CDKN2B*, two genes with a role in cell cycle inhibition and tumor suppression, may explain a causal association with cancer, the 9p21 locus does not contain described genes for CAD, and is not linked with major cardiovascular risk factors, such as plasma lipoproteins, and hypertension. As mentioned before, several studies, but not all, have found the association of this locus with type 2 DM (60–62, 64, 65). In this regard, it has been reported that susceptibility to CAD and diabetes is encoded by distinct, tightly linked SNPs on chromosome 9p21, thereby sustaining an independent association, with the ANRIL locus, of CAD and type 2 DM susceptibility (66). On the other hand, the putative molecular

TABLE 2 | Genes whose variants are commonly associated with both type 2 diabetes mellitus and cardiovascular disease.

Gene	Relative protein function	Role of genetic variant(s)	Reference
Adiponectin	Adipokine with anti-inflammatory and antiatherogenic effects	↑ Risk	(38)
ADIPOR1	Adiponectin receptor. Metabolism of fatty acids and glucose	↑ Risk	(39, 40)
ApoE	Lipoprotein transport	↑ Risk	(41, 42)
CDKN2A/2B	Cyclin-dependent kinase inhibitor. Cell cycle regulation	↑ Risk	(43)
CELSR2-PSRC1-SORT1	CELSR2 is part of the cadherin superfamily, involved in contact-mediated communication. Proline- and serine-rich coiled-coil 1 plays an important role in mitosis. Sortilin 1 plays a role in the trafficking of different proteins to either cell surface or subcellular compartments	↓ Risk	(43)
GLUL	Enzyme implicated in ammonia and glutamate detoxification, acid–base homeostasis, cell signaling, and cell proliferation	↑ Risk	(44, 45)
HMGA1	High-mobility group A1, architectural transcription factor with a role in cell growth, differentiation, and glucose metabolism	↑ Risk	(26, 27)
HNF1A	Hepatic nuclear factor 1A, involved in development and metabolic homeostasis	↑ Risk	(43)
HP	Haptoglobin. Hemoglobin-binding capacity. Implicated in angiogenesis and in cholesterol-crystallization-promoting activity	↑ Risk	(46, 47)
Paraoxonase	Enzyme that protects against lipid oxidation	↑ Risk	(48–51)
PCSK9	Proprotein convertase subtilisin/Kexin type 9. Plasma cholesterol metabolism	↓ Risk	(43)
PHACTR1	Phosphatase and actin regulator 1. PHACTR1 binds actin and plays a role in the reorganization of the actin cytoskeleton	↑ Risk	(43)
SOD2	Superoxide dismutase 2 transforms toxic superoxide into hydrogen peroxide and diatomic oxygen	↑ Risk	(52)
TCF7L2	Transcription factor 7-like 2, a member of the Wnt signaling pathway	↑ Risk	(40, 53, 54)

role of this locus in human CVD and type 2 DM has not been yet definitively identified. In fact, while mice lacking the orthologous region on chromosome 4 showed a reduction in cdkn2a and cdkn2b expression in several tissues, as well as increased incidence of cancers and increased proliferation of vascular smooth muscle cells (VSMCs), this condition was not associated with accelerated atherosclerosis (67). Moreover, studies aimed at evaluating CDKN2A/2B and lncANRIL levels in patients have provided conflicting data (68–70), underlying our current limit to interpret results from the non-coding genome. Recently, it has been hypothesized that the regulation of *CDKN2B* gene expression by lncANRIL could be involved in glucose homeostasis (71), while in diabetic patients, high glucose could alter ANRIL expression, favoring cell adhesion and cell proliferation, thereby leading to atherosclerosis (72). Other molecular mechanisms through which lncANRIL are associated with diabetes and its cardiovascular complications, however, remain unclear.

In another important study, 12 loci, previously identified by GWAS as predictors of coronary heart disease (CHD) in the general population, were investigated in three CHD case–control studies of diabetic patients. Among them, five variants, rs4977574 (CDKN2A/2B), rs12526453 (PHACTR1), rs646776 (CELSR2-PSRC1-SORT1), rs2259816 (HNF1A), and rs11206510 (PCSK9), showed a significant association with the risk for CHD also in type 2 DM (43). Among the type 2 DM susceptibility genes investigated by GWAS, the transcription factor 7-like 2 gene *(TCF7L2)* has been identified as one of the most significant (73). *TCF7L2* variants have been found to be associated with CVD in some (40, 53), but not in all (74) reports, although the association between *TCF7L2* risk alleles and CAD was not higher in diabetic individuals. Subsequent studies analyzed the association of three *TCF7L2* variants (rs7903146, rs12255372, and rs11196205) with CAD in 1,650 patients that underwent coronary angiography, and found that these variants were more strongly associated with CAD in diabetic patients than in non-diabetics (54).

Other genetic variants may confer more CHD risk in patients with type 2 DM than in non-diabetic subjects. An example is a polymorphism in the promoter region (−308) of the *TNF-α* gene, whose association with type 2 DM is even stronger in diabetic women (75). Also, as the apolipoprotein E (apo E) polymorphisms are known to modulate the risk for CVD in type 2 DM, many studies, but not all, have shown that the ApoE4 allele is related to a greater susceptibility for CVD in the presence of type 2 DM (41, 42). Another important challenge refers to the identification of diabetes-specific susceptibility genes for CVD. In this regard, interesting studies have addressed the haptoglobin (*HP*) gene polymorphisms. HP is a serum protein that binds free hemoglobin, and prevents hemoglobin-induced oxidation. It is synthesized by two alleles, *HP1* and *HP2*, the former encoded by 5 exons, and the latter by 7 exons, obtained by the intragenic duplications of exons 3 and 4. No significant association was shown between HP phenotype and CVD risk, whereas the *HP2* allele is strongly related to CVD in type 2 DM patients (46). The molecular explanations that may justify this specific association include the reduced ability of HP2, with respect to HP1, to prevent the oxidative stress driven by glycated hemoglobin (46, 76). Further studies have demonstrated that, in a large, type 2 DM-enriched cohort of Americans of European ancestry, the HP2-2 phenotype significantly associates with CVD mortality, triglyceride levels, and subclinical atherosclerosis, in the form of increased carotid-media thickness, but not of calcified arterial plaques (47). Also, a recent GWAS investigated the link between glutamate-ammonia ligase (*GLUL*) gene polymorphism and CHD, demonstrating that the association was specific for type 2 DM patients (44). Further studies confirmed the association of the rs10911021 *GLUL* variant with type 2 DM, and demonstrated that this polymorphism does not affect amino acid metabolism. However, although apparently counterintuitive, it is associated with lower HDL cholesterol levels, and large HDL particles (45).

These and other examples of type 2 DM-specific associated variants, while enriching our knowledge about CVD risk factors, contribute to the debate about the "common soil" hypothesis for type 2 DM and CVD (20, 77). In this context, only few significant loci for type 2 DM and CVD, identified by large-scale GWAS, had shown to be shared between both diseases. Starting from this provocative observation, new strategies have been used to identify novel and ethnic-specific genetic links between CVD and type 2 DM. For example, studies have been carried out using an integrative pathway and network analysis combined with GWAS in more than 15,000 women from three different ethnicities, leading to the identification of eight major pathways shared by type 2 DM and CVD in all ethnic groups (78). In these studies, key driver genes, influencing the extra-cellular matrix composition, such as *COL1A1*, *COL3A1*, and *ELN*, that had been cross-validated in mouse models for type 2 DM and CVD, have also emerged. Interestingly, few peculiar pathways related to specific ethnic groups were identified (78). In addition, in the past years, attempts have been made to assess a more reliable disease susceptibility for CVD in type 2 DM by analyzing cumulative genetic risk from multiple loci rather than from single SNPs (79, 80). As an example, two genetic risk scores have been successfully used to predict CVD and CVD fatal outcomes using patients from the Diabetes Heart Study (81).

HMGA1: An Established Gene for Type 2 DM Risk and a Novel Gene Predisposing to MI

High-mobility group A1 is a small, non-histonic nuclear protein, with pleiotropic effects involved in the regulation of embryogenesis, oncogenesis and tumor progression, cell differentiation, as well as inflammation (82–84). As an architectural transcription factor, it binds to the minor groove of AT-rich regions of DNA, and alters the chromatin conformation, facilitating the assembly and stability of stereospecific DNA–protein complexes called "enhanceosomes," which drive gene transcription (85–87). Many studies from our group have demonstrated the role of HMGA1 in the transcriptional control of glucose metabolism, being a key regulator of the insulin receptor (*INSR*), insulin-like growth factor binding protein 1 (*IGFBP1)*, retinol binding-protein 4 (*RBP4*), *visfatin*, and insulin (*INS*) genes (88–93), as well as an important mediator of insulin action (94). Defects in HMGA1 protein, or the association with functional *HMGA1* variants, among which the most common rs139876191 variant (previously named

rs146052672), cause a decrease in *INSR* expression and a trans-ethnic increased susceptibility to either type 2 DM (26, 95–98) or metabolic syndrome (99). Besides its effects on glucose homeostasis, HMGA1 plays a role in adipogenesis and lipid metabolism (100–102), while the HMGA1 rs139876191 variant correlates with body mass index, and reduced HDL levels in patients with metabolic syndrome and type 2 DM (97, 99).

Also, HMGA1 plays a critical role in the development and progression of the atherosclerotic plaque by promoting the proliferation and the migration of VSMCs to the neointima, and by inducing the expression of several inflammatory cytokines, adhesion molecules, including CD44, and chemokines (103, 104). On the other hand, by activating the matrix metalloproteinase 9 (MMP-9), and the vascular endothelial growth factor (VEGF), HMGA1 is essential for vascular repair and neoangiogenesis, whereas its lack causes impairment of both vascular protection from injuries and of neovascularization (92, 105, 106). Recently, the functional HMGA1 rs139876191 variant has been found to be associated with acute MI, independently of type 2 DM or other cardiovascular risk factors, such as hypertension, obesity, and gender, suggesting that *HMGA1* may represent a new candidate gene for acute MI and a marker for cardiovascular risk (27). Although further studies in other populations are needed to confirm this association, due to its pathophysiological role in insulin resistance, glucose homeostasis, lipid metabolism, inflammation and vascular repair, HMGA1 may represent a convincing molecular link between type 2 DM and MI.

EPIGENETIC CHANGES

Epigenetic processes are defined as heritable modifications in gene expression that occur in the absence of changes in the DNA sequence, and include DNA methylation, histone acetylation, and RNA-based mechanisms. These processes are cell-specific, susceptible to modifications, and responsive to the environment, and should be taken into account to better understand otherwise hidden causes of diseases.

DNA or Histone Modifications

New research investigations have addressed the link between epigenetic factors, type 2 DM and CVD. Hyperglycemia, for example, can induce epigenetic changes that lead to the overexpression of genes implicated in vascular inflammation. In particular, hyperglycemia has been shown to activate the NF-kB signaling pathway in cultured THP-1 monocytes, leading to the production of MCP-1 and other inflammatory factors, and to the expression of adhesion molecules in endothelial cells, providing a plausible molecular mechanism for endothelial dysfunction and atherosclerosis (107). On the other hand, clinical studies have demonstrated that early intensive control of glycemia in diabetic patients is crucial to prevent chronic micro- and macrovascular complications, reinforcing the notion that glycemia may have a longstanding influence on clinical outcomes, a phenomenon called "metabolic memory" (108).

In support of an epigenetic role of hyperglycemia, it has been demonstrated, in aortic endothelial cells, that exposure to high glucose correlates with the inverse acetylation of the histone H3K9/K14 and modified DNA methylation patterns (109). Several histone lysine modifications have also been described following transient high glucose levels that may account for a persistent transcriptional induction of the *RELA* gene, encoding for the p65 subunit of NF-kB, even after subsequent incubation of endothelial cells with normal glucose concentrations (110). Altogether, the net result of this activity leads to the transcriptional activation of some target genes implicated in the endothelial dysfunction, and the repression of other ones (111). Acetylation or hyperacetylation may also occur, being responsible for the increased expression of *HMOX1*, *MMP10*, *SLC7A11*, *MMP1*, *MCP-1*, and *ICAM* genes (109). Hyperglycemia is, however, not the only inducer of epigenetic modifications. Many other pathophysiologic mechanisms that may be operative in diabetes, independently from glucotoxicity, like ROS, PKC activation, and AGEs have been described to induce also epigenetic changes (112). In particular, ROS production is able to significantly induce the CpG hypomethylation of the $p66^{Shc}$ promoter and, at the same time, an increment in the H3 histone acetylation. Thus, ROS-induced epigenetic modifications are associated with higher levels of $p66^{Shc}$, a mitochondrial adaptor that modulates the intracellular redox state, and with significant activation of PKC, therefore sustaining endothelial dysfunction and vascular damages (111, 112).

Further studies have investigated the associations between epigenetic modifications and cardio-metabolic phenotypes, such as obesity, dyslipidemia, insulin resistance, inflammation, and hypertension, in relation to CVD risk (113). In a recent study, peripheral blood mononuclear cells were used to measure histone deacetylases (HDACs) activity and expression in relation to glycemia, inflammation and insulin resistance in patients with type 2 DM. Low-grade chronic inflammation and insulin resistance induced HDAC3 activity and expression, and correlated positively with circulating levels of TNF-α, IL-6, and other proinflammatory markers, and negatively with Sirt1 expression (114).

Several reports have demonstrated a correlation between DNA methylation and cardiovascular risk. The susceptibility haplotype rs8050136 of the *FTO* gene, a prominent gene associated with increased risk for obesity and CVD, displayed increased levels of methylation (115); a similar mechanism has been hypothesized for the rs9939609 polymorphism (116). In another candidate gene study, an association between IGF2 methylation and lipid profile alterations was found in obese children. In particular, IGF2 hypermethylation was associated with higher triglyceride/HDL-cholesterol ratio, representing an epigenetic marker of metabolic risk (117). Another study that combined genome-wide transcriptome and CpG methylation profiling by array, reported many differentially methylated predicted sites in adipose tissue from insulin-resistant patients compared to controls, which included genes involved in insulin signaling and in the interaction with integrins (118). Altered methylation were also found in *IL18, CD44, CD48, CD38, Cd37, CX3CL1, CXCR1, CXCR2, CXCL1, IGF1R, APOB48R, LEF1, GIPR, GRB10, SIRT2, HDAC4, DNMT3A, LEPR*, and *LEP* genes that were already found to be strongly and independently associated with insulin resistance (118–121). In addition, polarization of adipose tissue

macrophages from an anti-inflammatory (M2) to a proinflammatory phenotype (M1) in obese mice was shown to involve the methylation of the PPARγ promoter (122). Finally, there are evidences that MI susceptibility risk may be influenced by epigenetic changes occurring in the prenatal environment (123).

Abnormalities in MicroRNA (miRNA) Expression

MicroRNAs are small single-strand RNA molecules that influence their target genes at a posttranscriptional level, thereby regulating many biological processes. Since their discovery about two decades ago, numerous miRNAs have been described to be associated with a multitude of diseases, including type 2 DM and CVD (28, 124, 125). In particular, with reference to type 2 DM, miRNAs have shown to be involved in regulating beta cell function, insulin response, glucose homeostasis, as well as the pathogenesis of diabetic vascular complications (126, 127). Research in this field has highlighted new mechanistic links between diabetes and CVD (128), with many evidences proving the involvement of distinct miRNAs in the pathological steps that lead to atherosclerosis (**Figure 1**).

In vitro and *in vivo* studies concerning the mechanisms that are responsible for the endothelial dysfunction in diabetes demonstrated that, in the presence of high glucose concentrations, upregulation of miR-185 reduced the expression of the glutathione peroxidase-1 (*GPx-1*) gene, which encodes an enzyme that is important in the prevention of oxidative stress (129); instead upregulation of miR-34a and miR-204 contributed to endothelial cell senescence by impairing SIRT-1 expression and function (130, 131). In the endothelium, miR-126 exerts proangiogenic, and anti-inflammatory activities. At a functional level, it enhances VEGF and fibroblast growth factor activities, contributing to vascular integrity and angiogenesis (132, 133), recruits progenitor cells through the chemokine CXCL12 (134), while it suppresses inflammation by inhibiting TNF-α, ROS, and NADPH oxidase *via* HMGB1 (135). Consistently, miR-126 levels are down-regulated in both myocardial tissue and plasma from type 2 diabetic patients without any known anamnestic data for CVD (136, 137), and in patients with CAD (138), suggesting that it could represent a new diagnostic marker for diabetes and CVD. Other studies in endothelial colony-forming cells, as well as in progenitor endothelial cells (EPCs) exposed to high glucose, demonstrated that miR-134 and miR-130a affected cell motility and apoptosis, respectively (139, 140).

In diabetes, VSMCs loose their contractility and acquire proliferative and migratory properties, facilitating the onset of pathological processes relevant to CVD (141). miR-145 has proved to reduce its level in the presence of high glucose, to impair *myocardin* gene expression *via* Klf4, and to facilitate VSMC proliferation (29, 142). In this context, a role of miR-504 and miR-24 in promoting VSMC proliferation and migration, has also been reported (143, 144).

An important issue is the link between lipid metabolism and miRNAs in diabetic CVD. Several important genes implicated in lipid synthesis or processing, like *FoxA2*, *Ppargcla*, *Hmgcs2*, and *Abdhd5* have been shown to be dysregulated by miR-29 in Zucker diabetic fatty rats (145), while HNF-4 alpha was found

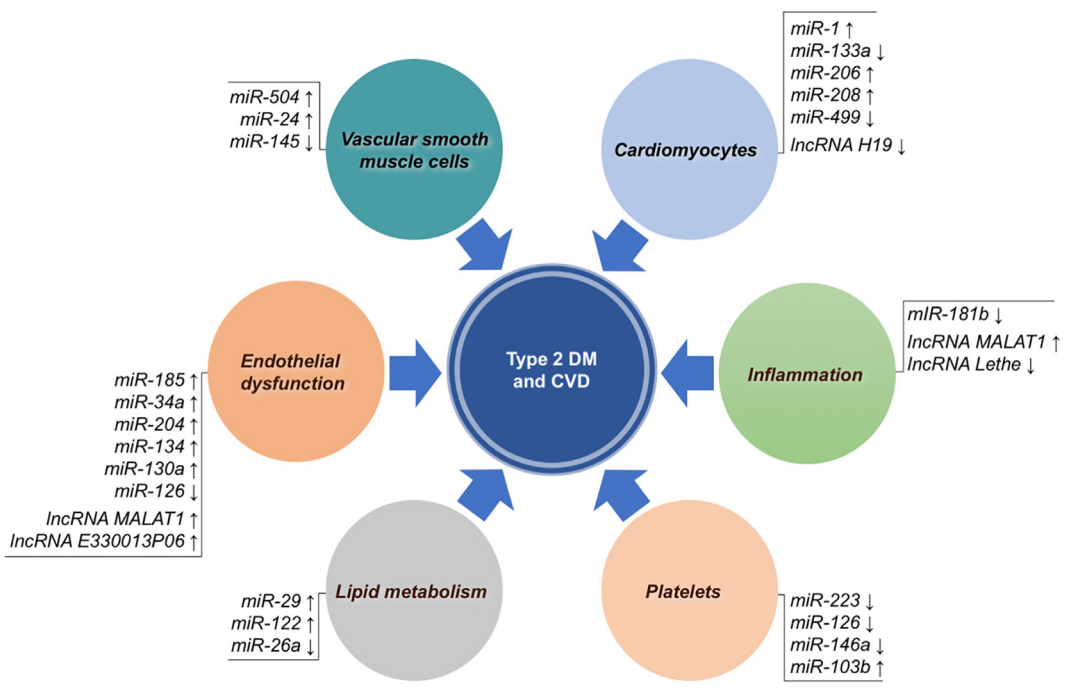

FIGURE 1 | Non-coding RNAs associated with both type 2 diabetes mellitus (DM) and cardiovascular disease (CVD). MicroRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are grouped according to their main biological mechanism involved in atherosclerotic CVD. Arrows indicate overexpression (↑) or underexpression (↓).

to be raised by increased levels of miR-122 in diabetic mice and insulin-resistant HepG2 cells (146). Both miR-122 and HNF-4 alpha were able to upregulate the expression of *SREBP-1* and *FAAS* genes, causing abnormal cholesterol homeostasis and high levels of fatty acid and triglyceride synthesis (146). Finally, decreased levels of miR-26a have been reported in obese mice, in which they contribute to increased fatty acid synthesis, and to obesity-related metabolic complications (147).

Platelets are key partaker in CVD and their involvement in the development of cardiovascular complications is strengthened in diabetes (148). Platelets play an important role in the pathophysiology of thrombosis and represent an important source of different RNA species, including pseudogenes, intronic transcripts, non-coding RNAs, and antisense transcripts (149, 150). These molecules can be released by platelets through microvescicles, contributing to the horizontal transfer of molecular signals delivered through the bloodstream to specific sites of action (151). The downregulation of miR-223, miR-126, or 146a observed in diabetic and hyperglycemic patients (137, 152) has been associated with increased platelet reactivity and aggregation (153, 154). In line with these findings, silencing of miR-223 in mice caused a hyperreactive and hyperadhesive platelet phenotype, and was associated with calpain activation through the increased expression of beta1 integrin, kindlin-3, and factor XIII (153, 155). Moreover, the modulation of the expression levels of platelet miRNAs can also be measured in plasma. In fact, plasma levels of miR-223 and miR-126 are decreased in diabetics (137, 156). This leads to the upregulation of the P2Y12 receptor, as well as P-selectin, further contributing to platelet dysfunction (156). As a result of this interaction, activation level of platelets in type 2 DM is increased (149, 156, 157). Consistently with this, circulating miR-223 levels are independent predictors of high on-treatment platelet reactivity (158). Another interesting mechanism linking platelets and diabetes involves miR-103b, a platelet-derived biomarker proposed for the early diagnosis of type 2 DM, and the secreted frizzled-related protein-4 (SFRP4), a potential biomarker of early β cell dysfunction and diabetes. In fact, platelet-derived miR-103b is able to downregulate SFRP4, whose expression levels are significantly increased in pancreatic islets and in the blood of patients with prediabetes or overt diabetes (159). These interesting results identify miR-103b as a novel potential marker of prediabetes and diabetes, and disclose a novel potential therapeutic target in type 2 DM.

Macrophages also play a key role in atherosclerotic plaques. Unbalanced production of proinflammatory molecules from adipose tissue contributes to the polarization of macrophages toward the M1 phenotype and their accumulation within the vessel wall (160, 161). It has been demonstrated *in vitro* and *in vivo* that in the presence of high glucose or in insulin-resistant states, endothelial cells decreased miR-181b expression, while the production of this miR, through the inhibition of AKT Ser 473 phosphorylation, was associated with a M2 anti-inflammatory response, but not with antiproliferative effects (162). These results are compatible with an inhibitory role of miR-181b in atherosclerosis.

Other miRNAs, abundantly expressed in cardiomyocytes, such as miR-1 and miR-133a, seem to be crucial in preventing myocardial dysfunction. Both these miRNAs have been shown to be reduced in ischemic myocardial tissue, in left ventricular hypertrophy, and in diabetic cardiomyopathy (163, 164). Among the molecular mechanisms proposed for miR-133a, the repression of serum response factor, which plays a role in myoblast proliferation, of RhoA (a protein involved in GDP-GTP cycling), Cdc42 (a kinase implicated in hypertrophy), and Nelf-A/WHSC2 nuclear factor (165).

Many cardiac-enriched miRNAs have been reported to be responsive to hyperglycemia, including miR133a, miR-1, and miR-206, with the last two favoring the apoptosis of cardiomyocytes through the negative regulation of the heath shock protein 60 (166). Recent evidences demonstrated that miR-208 and miR-499, together with miR-1 and miR-133, could play a role into the molecular mechanisms leading to the differentiation of stem cells into cardiomyocytes (167). In fact, the involvement of miR-133a in the modulation of contractility was recently demonstrated in streptozotocin-induced diabetic rats (168), in which miR-133a overexpression was able to improve contractility through the upregulation of tyrosine aminotransferase, a known regulator of norepinephrine production and β-adrenergic receptors (168). These latter findings are particularly interesting, as we could recently demonstrate that miR-133a transcoronary concentration has an interesting prognostic potential in patients with CVD (169). Less data is currently available on the involvement of miR-208 in diabetic heart disease. A proposed mechanism for this miRNA implicated a role in the regulation of myosin heavy chain gene expression (170). On the other hand, functional studies showed that miR-499 protects cardiomyocytes from ischemic damage and apoptosis *via* the suppression of calcineurin-mediated dephosphorylation of dynamin-related protein-1 (171).

Specific miRNAs, such as miR-15, -16, -26a, -196a2, and Let-7a (172) are able to modulate HMGA1, whose association with acute MI, type 2 DM, and cardiovascular risk has already been discussed (26, 27, 99). Also, HMGA1 can specifically induce the expression of miR-10b, -21, -125b, -221, -222, or inhibit the production of miR-34a and -603, all of which are involved in several aspects of cardiovascular pathophysiology (173), thereby further supporting the notion that a complex relationship indeed exists between HMGA1 and miRNAs in this context (29, 174).

Abnormalities in Long Non-Coding RNA (lncRNAs) Expression

Long non-coding RNAs include non-protein coding transcripts longer than 200 nucleotides (175, 176). They have both nuclear and cytoplasmic location and work as signal amplifiers for biological activity, regulating gene expression through a variety of partly explored molecular mechanisms, including the interaction or competition with other RNAs, DNA binding proteins, and specific regulatory DNA sequences (176, 177). New increasing evidences show the involvement of lncRNAs in human diseases (178), such as cardiometabolic diseases (179–182). For example, in the context of atherosclerosis (**Figure 1**), experimental studies have shown altered expression of lncRNAs in several processes implicated in SMC proliferation, endothelial function, inflammatory cells, lipid metabolism and obesity, as well as with insulin

resistance (183), while clinical studies have demonstrated that circulating lncRNAs could be potentially used to predict type 2 DM (182) or the outcome of heart failure (184). However, data from this kind of studies are still initial and in progress. The first lncRNA robustly associated with CVD and type 2 DM has been lncANRIL, a locus identified by GWA studies, already widely discussed in this review in the Section "Genetic Polymorphisms." Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is an lncRNA particularly expressed in the nucleus and physiologically implicated in the regulation of endothelial cell function. It has been demonstrated that hyperglicemia alters MALAT1 expression, leading to micro- and macrovascular damages (185–187). In particular, at a molecular level, MALAT1, by targeting serum amyloid antigen 3, a proinflammatory ligand, has been shown to induce the expression of IL-6 and TNF-α, as well as ROS production, thereby promoting endothelial dysfunction (187). Recently, the lncRNA H19, which has a role in limiting body weight and cell proliferation, was found to be markedly reduced in a mouse model of diabetic cardiomyopathy as a consequence of hyperglycemia (188). In an elegant study, it was demonstrated that lncRNA H19, *via* mIR-675, targets VDAC1, a mitochondrial porin that plays a role in ATP transport, regulating cardiomyocyte apoptosis (188). In other cases, lnc RNAs have been implicated in diabetic vascular complications through mechanisms linked to macrophage-mediated inflammation. By transcriptome profiling of bone marrow-derived macrophages from db/db and diet-induced insulin-resistant type 2 diabetic mice, an increase in lncRNA E330013P06 has been observed, demonstrating that this lncRNA promoted foam cell formation and endothelial dysfunction through the expression of inflammatory genes like *Nos2*, *IL6* and *ptgs2* (189). Also, a recent study using RAW264.7, as well as bone-derived macrophages, showed that lncRNA Lethe exerted an anti-inflammatory role by inhibiting the translocation of NF-kB transcription factor to the nucleus, and that in the presence of high glucose concentrations, lncRNA Lethe expression was reduced, with a consequent increment in *NOX2* gene expression and ROS production (190).

CONCLUSION

In this review, we provide an overlook about the main genetic and epigenetic factors linking type 2 DM and CVD, with a particular emphasis on the pathophysiological mechanisms involved. We addressed known genetic variants shared by both conditions, and the most relevant epigenetic mechanisms involved in their interplay. However, as a lower amount of solid evidence is available to date about epigenetics in this pathophysiological context, further research will be necessary to validate, in patients with type 2 DM, the results obtained so far *in vitro* and *in vivo*, in animal models. A deeper understanding of gene networks, intracellular pathways, and cell-to-cell communication mechanisms will allow the identification of novel biomarkers, as well as new therapeutic targets to exploit in the management of CVD in patients with type 2 DM.

AUTHOR CONTRIBUTIONS

SR and BA prepared the first draft of the manuscript; EC was involved in the literature search; AB, CI, and DPF critically revised the manuscript and wrote the final version of the article.

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REVIEW
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The Potential Role of Platelet-Related microRNAs in the Development of Cardiovascular Events in High-Risk Populations, Including Diabetic Patients: A Review

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Platelet activation plays a pivotal role in the development and progression of atherosclerosis, which often leads to potentially fatal ischemic events at later stages of the disease. Platelets and platelet microvesicles (PMVs) contain large amounts of microRNA (miRNA), which contributes largely to the pool of circulating miRNAs. Hence, they represent a promising option for the development of innovative diagnostic biomarkers, that can be specific for the underlying etiology. Circulating miRNAs can be responsible for intracellular communication and may have a biological effect on target cells. As miRNAs associated to both cardiovascular diseases (CVD) and diabetes mellitus can be measured by means of a wide array of techniques, they can be exploited as an innovative class of smart disease biomarkers. In this manuscript, we provide an outline of miRNAs associated with platelet function and reactivity (miR-223, miR-126, miR-197, miR-191, miR-21, miR-150, miR-155, miR-140, miR-96, miR-98) that should be evaluated as novel biomarkers to improve diagnostics and treatment of CVD.

Keywords: biomarker, microRNA, platelet microvesicles, platelet reactivity, cardiovascular diseases, type 2 diabetes mellitus

INTRODUCTION

Platelets largely contribute to the progression of atherosclerosis and the development of its clinical complications (1, 2). Upon platelet adhesion to damaged loci of blood vessel walls, at sites of endothelial cell activation, they promote the growth of chronic atherosclerotic plaques, and precipitate the onset of arterial thrombosis following atherosclerotic plaque rupture (3). In addition, platelet activation can induce and maintain a local pro-atherothrombotic mileu, through specific alterations of the arterial wall (4). Platelets are therefore the key cellular component of athero-thrombosis. Notwithstanding their major impact on the development of potentially fatal ischemic events in late phases of cardiovascular diseases (CVD), several aspects of the underlying molecular mechanisms driving platelet activation are yet to be fully clairified (5). Type 2 diabetes mellitus (T2DM) represents a dangerous threat to health worldwide, and up to 50–75% of deaths are due to its macrovascular

complications (6, 7). In line with previous reports, platelet reactivity is a critical contributing element to the development of cardiovascular complications in T2DM population (6).

Even though platelets are devoid of nucleus and genomic DNA, they have the capacity to translate inherited messenger RNA (mRNA) into protein. In fact, a strong relationship was demonstrated between transcriptome and proteome in platelets (8, 9). The combination of several distinctive features possibly enables posttranscriptional modulation of gene expression within platelets. Since they are equipped with a complex translational apparatus, as well as unique transcriptome and correlating proteomic profile, they have the capacity to sustain *de novo* translation (10). Interestingly, in recent years it was reported that microRNAs (miRNAs) do not act exclusively on the intracellular level but they also exert their influence extracellularly (11). Exosomes, which represent compact plasma membrane-derived vesicles released by numerous cell types into the extracellular space, function as intercellular signaling molecules (12). They accommodate bioactive proteins, lipids, DNA, mRNAs, and miRNAs carrying significant biological information and deliver them to specific recipient cells (13, 14). Exosomes are able to carry distinct quantities of miRNAs that transfer biological information inbetween cells (12). Upon activation, platelets secrete microvesicles (MVs) containing growth factors and several effector proteins, as well as multivesicular bodies and exosomes, which are able to exert extracellular effects (10, 15). Platelet microvesicles (PMVs) play a role in maintenance of hemostasis, vascular health, and immunity; however, they are also involved in thrombotic and inflammatory disturbances (15). Most importantly, PMVs represent intercellular carriers of Ago2—miRNA complexes, such as miR-223, that exert regulation of gene expression in endothelial cells. This response is considered pro-inflammatory and likely to contribute to the development of cardiovascular events (16).

microRNAs are small, endogenous, noncoding RNAs (17). They regulate a significant proportion of protein coding genes through the interaction with mRNAs (17, 18). This effect is exerted by binding corresponding parts of mRNA transcripts to suppress their translation and control degradation (19).

Until now, 1,881 miRNA sequences have been listed for *Homo sapiens* (20). However, in a recent analysis, less than 20% of human miRNAs met the criteria of high confidence for miRNAs annotation, based on deep sequencing technology (21). Since miRNAs can be easily measured by real-time polymerase chain reactions (PCR) in plasma or serum, they have attracted special interest as potential novel biomarkers and instrument to discover the process of platelet gene expression (22). They provide opportunities to study, observe, as well as control platelet function and vascular condition in patients with potentially higher risk of developing cardiovascular events. Moreover, circulating miRNAs may reflect platelet activation, and therefore, may serve as a substitute marker of efficacy of antiplatelet therapy. However, further studies should be designed to elucidate the mechanism and investigate the associations between miRNA and cardiovascular diseases.

In this systematic review, we present an overview of current knowledge on diagnostic and prognostic value of miRNAs related to platelets in patients with CVD and/or T2DM.

ARTICLE SEARCH PROCESS

The electronic databases PubMed and Scopus were searched through October 23rd, 2017 for studies that evaluated potential prognostic role of miRNA associated with platelet reactivity in diabetics, using the following search syntax: "Search ("micrornas" [MeSH Terms] OR "mir" [MeSH Terms] OR "mirna" [MeSH Terms]) AND ("platelets" [MeSH Terms] OR "platelet activation" [All Fields] OR "platelet aggregation" [All Fields]) AND ("diabetes mellitus" [MeSH Terms] OR "diabetes" [All Fields]) AND ("prognosis" [MeSH Terms] OR "prognosis" [All Fields]) Filters: Humans. Our search was limited to human studies and did not exclude studies based on ethnicity of study participants. A total of 50 records were identified after duplicates removal. Titles and abstracts were screened by two independent operators, with exclusion of 26 records for any of the following reasons: (a) they were not related to the specific research question ($n = 6$); (b) they did not present original data ($n = 18$); they were not human studies ($n = 2$). Finally, 24 articles were selected to be used in this review. **Figure 1** reports the article selection flowchart.

MOST ABUNDANT miRNAs IN PLATELETS

Platelets express high levels of miRNAs. miR-223, miR-126, miR-197, miR-24, and miR-21 represent the most abundant miRNAs in human platelets and PMVs, as shown during microarray screening (10). Moreover, flow cytometry showed that circulating miRNA levels correspond with PMVs level (10). Interestingly, these miRNAs have been reported to correlate with CVD and are emerging as potential biomarkers for risk assessment in CVD and monitoring of antiplatelet drug efficacy (23, 24). Specific miRNA signatures had already been identified to be specifically associated to T2DM and could therefore be exploited as biomarkers of this disease in the future (see **Figure 1**) (25–31). As novel biomarkers and technologies arrive at the horizon, the use of platelet miRNA testing in CVD and T2DM appears to take on a new aspect.

miR-223

miR-223, richly expressed in platelets and megakaryocytes, is involved in the development of the hematopoietic lineage (5). The gene encoding miR-223 is on the X chromosome (8). In a landmark study, Landry et al. confirmed that human platelets contain a plentiful array of miRNAs, with miR-223 being one of the most abundant ones (32). Similar evidence was independently reported by another group (33).

Despite the fact that multiple reports have been published on miRNAs thus far, the information on their function in platelets is still scarce. Landry et al. also found that miR-223 targets the adenosine diphosphate (ADP)-receptor P2Y12, a purinergic receptor known to have an impact on platelet reactivity, by amplifying aggregation induced by all known platelet agonists. By identifying the binding site for miR-223 on the P2Y12 mRNA, as well as demonstrating the capacity of miR-223 to control gene expression of platelet precursor cells, they provided a relevant piece of information supporting the hypothesis that P2Y12 expression is regulated by miRNAs in human platelets (8, 32, 34).

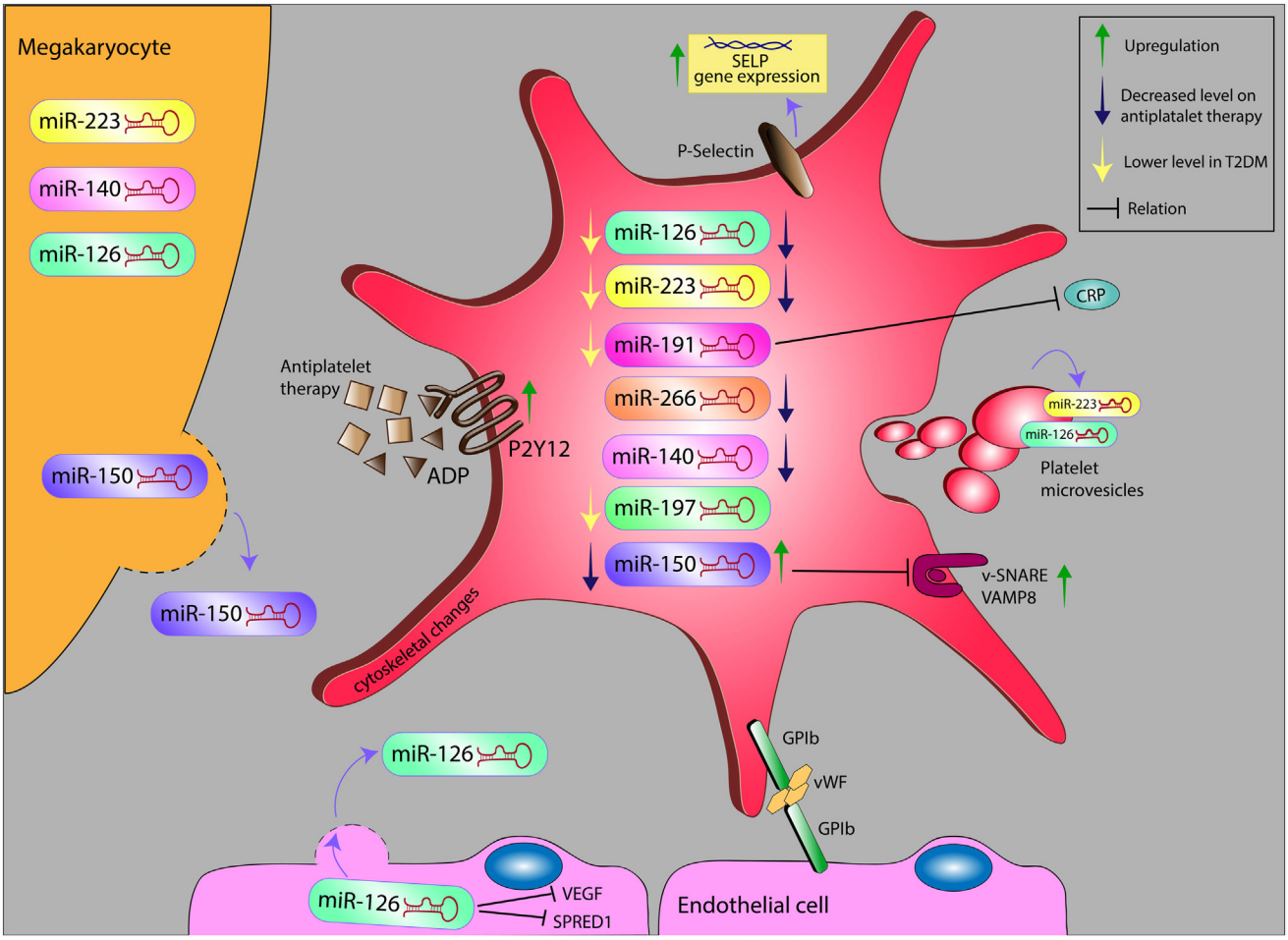

FIGURE 1 | Alteration levels of miR in platelet and platelet microvesicles, and their possible relation with inflammatory markers on antiplatelet therapy in diabetes. miR, microRNA; ADP, adenosine diphosphate; VEGF, vascular endothelial growth factor; SPRED1, sprout-related EVH1 domain-containing protein 1; GP1b, glycoprotein Ib; vWF, von Willebrand factor; SELP, selectin P; VAMP8, vesicle-associated membrane protein 8; CRP, C-reactive protein.

Moreover, Nagalla and colleagues revealed that miRNA profiles are linked to, and may predict, the response of platelet aggregation to epinephrine (33). Several studies were then conducted to validate the concept that plasma levels of miR-223 become lower in patients treated with antiplatelet therapy (19, 22, 35, 36). According to Shi et al., platelet miR-223 downregulation correlated with a weaker response to P2Y12 receptor antagonist clopidogrel in a population of 33 Chinese patients (19). The link between circulating miR-223 levels and responsiveness to clopidogrel in patients with coronary heart disease (CHD) was further investigated by Zhang et al. During an analysis of 62 patients with troponin-negative non-ST segment elevation acute coronary syndrome (NSTE-ACS) they found that a decrease in circulating levels of miR-223 was the only independent predictor for platelet reactivity index (PRI)-determined lower responders (OR 0.111, 95% CI: 0.018–0.692, $P = 0.019$), even though it was tested among known predictors of platelet reactivity (e.g., CYP2C19*2/*3 loss-of-function genotypes, use of calcium channel blockers/proton-pump inhibitors, age, diabetes, smoking) (35). On the contrary, results obtained by Chyrchel et al. in 21 males with CHD argued against the hypothesis that plasma levels of miR-223 mark platelet responsiveness to DAPT. More potent platelet inhibition related predominantly to novel P2Y12 antagonists appeared to coexist with higher miR-223 compared to subjects with diminished responsiveness to DAPT (36). Recently, Kaudewitz et al. reported that antiplatelet therapy decreases plasma levels of platelet miRNAs, including miR-223. In a cohort of 125 patients with a previous acute coronary syndrome (ACS), the key platelet-related miR-223 was correlated with platelet function tests ($r_p = 0.28$; $n = 121$; $P = 0.002$) (18). The emerging evidence of more effective platelet inhibition, resulting in decrease of miR-223, highlights the potential role of this miRNA in monitoring the process of platelet activation and efficacy of antiplatelet therapy (1).

To date, three studies analyzed the association of miR-223 with clinical endpoints (37–39). Schulte evaluated 873 individuals and revealed that elevated miR-223 levels reliably predicted future cardiovascular deaths [HR 2.23 per one SD increase (1.20; 4.14), $P = 0.011$, C-index 0.80], as 2.1% ($n = 18$) of the subject

experienced cardiovascular death over a median follow-up time of 4 years (37). Also Zampetaki and colleagues investigated the link between baseline miRNA quantity and incident myocardial infarction in a population of 820 patients and found that miR-223 was negatively related to disease risk (38). More recently, Keller et al. analyzed a panel of miRNAs to predict outcome in the context of cardiovascular disease prevention. They determined that all-cause 5-year mortality was associated with lower miR-223 levels (HR: 0.30; 95% CI: 0.08–1.07; $P = 0.063$) (39). The presented results show that miR-223 may not only serve as a biomarker of platelet activation, but could also be exploited as a prognostic marker.

Interestingly, lower plasma levels of miR-223 were found in T2DM (25, 34, 40). In particular, miR-223, together with miR-126, miR-140, and miR-26b are expressed at a lower level in both platelets and megakaryocytes from T2DM patients, leading to upregulation of P2Y12 receptor and SELP (P-selectin), thus contributing to platelet hyperactivation (34). In accordance with these findings, a pilot study demonstrated that hyperglycemia-associated downregulation of miR-223 and miR-146a mediates platelet activation in diabetics, favoring ischemic stroke (40). Furthermore, circulating levels of miR-223 were found to independently predict the response to clopidogrel treatment, which shows potential use of this miRNA in the assessment of efficacy of antiplatelet therapy (35).

Altogether, miR-223's diverse and complex regulatory functions suggest this miRNA might be used as a potential biomarker of platelet activation, a surrogate marker of antiplatelet treatment efficacy, as well as predictor for the risk of cardiovascular death and a tool in T2DM diagnosis.

miR-126

Another important miRNA associated with platelet function, miR-126, is located on human chromosome 9 (5). Both strands of miR-126: miR-126-3p and miR-126-5p, are biologically active (41). miR-126 belongs to the most abundantly expressed miRNAs in endothelial cells being responsible for vascular development, integrity, and response to hemodynamic stress (25).

Although miR-126 has been linked to angiogenesis and to the development of CVD in several independent reports, few studies focused on its role in platelet activation (18, 25, 42). Willeit et al. revealed that platelet inhibition through administration of antiplatelet drugs [10 mg prasugrel or 10 mg prasugrel with 75 mg acetylsalicylic acid (ASA)] resulted in reduction of miR-126 levels (1). Moreover, de Boer et al. confirmed the correlation between the concentration of miR-126 in plasma and platelet activation *in vivo*. In fact, ASA administration resulted in reduction of circulating platelet-derived miR-126 in patients with T2DM (42). Kaudewitz and coworkers independently confirmed that alterations in miR-126 affect platelet reactivity (18). These results underline the key role of miR-126 in platelet activation.

Furthermore, Zampetaki et al. suggested that alterations in circulating miR-126 levels have a diagnostic and prognostic value as a biomarker for endothelial dysfunction in T2DM (43). In another study by Olivieri et al., the expression of miR-126-3p in healthy controls was markedly increasing with their age, what was paralleled by a raise of intra/extracellular miR-126-3p in senescent *in vitro* cultured human endothelial cells (HUVECs) (44). Interestingly, such age-related differences in miR-126-3p plasma levels were not observed in T2DM patients. On the other hand, when compared with age-matched controls or T2DM patients with appropriate glycemia, miR-126-3p levels were lower in T2DM patients with poor glycemic control. In an *in vitro* model, miR-126-3p expression in HUVECs cultured in high glucose medium was significantly lower than in HUVECs exposed to low glucose concentration. These results indicate that miR-126-3p might be evaluated as a biomarker of physiological senescence of endothelial cells in patients with appropriate glycemia level, but also for impaired survival of endothelial senescent cells exposed to high glucose levels in T2DM patients (44). Stratz et al. assessed platelet miRNA profiles in a cohort of 60 patients, including clinically stable diabetic, and non-diabetic patients, and no significant differences in plasma miR-126-3p between diabetic and non-diabetic patients were noted (45). Zampetaki et al. sought to evaluate miRNA profiles in diabetic subjects and a potential association between miRNA expression and MI. High glucose concentration resulted in significantly reduced miR-126 amount in endothelial apoptotic bodies, followed by reduced miR-126 plasma level. Since miR-126 facilitates VEGF signaling by repressing SPRED1 and PIK3R2/p85-β, it has been suggested that low plasma miR-126 levels might have an impact on VEGF resistance and endothelial dysfunction in patients with T2DM (25). Furthermore, moderate decrease in miR-126 levels in normal glucose, impaired fasting glucose/impaired glucose tolerance and T2DM was observed (43). Hence, the decreased level of miRNAs that are highly expressed in platelets, such as miR-126, may reflect platelet dysfunction in the diabetic population.

miR-126 has been proposed as a candidate biomarker of cardiac diseases, as it was positively correlated with incident MI (38). Prognostic values of several miRNAs (miR-126, miR-21, miR-130, miR-222, miR-20a, miR-let7d, miR-27a, miR-92a, miR-17, miR-199a) for the occurrence of cardiovascular events in patients with stable coronary disease were reported in later studies (46, 47). In particular, Jansen et al. reported no significant association between cardiovascular events (median follow-up period was 6.1 years) and plasma levels of the selected miRNAs. Nonetheless, among the miRNAs tested, increased levels of miR-126 in circulating microvesicles were reported to correlate with lower predisposition to major adverse events in patients with stable coronary artery disease (CAD) (46). The positive effect of miR-126 on cardiovascular system was established by Harris and colleagues, who proved that the level of endogenous miR-126 is negatively associated with VCAM-1 expression. As a result, alterations in miR-126 expression control vascular inflammation by influencing leukocyte adhesion to endothelium (48). de Rosa et al., on the other hand, measured miR-126 concentration in patients undergoing coronary angiography who were separated into 3 groups depending on evidence of coronary artery disease and troponin (hsTNT) levels. The samples were obtained from both coronary venous sinus and aorta. They revealed that miR-126 level in aorta was positively correlated with hsTNT concentration. Interestingly, only in patients with acute coronary syndrome miR-126 concentration in CVS was lower than in the

aorta, suggesting consumption of endothelial miR-126 during transcoronary passage (49).

However, the administration of antiplatelet drugs should be considered when using circulating miR-126 and possibly other platelet-derived miRNAs as diagnostic biomarker for CVD (42). Carino et al. showed that the change of antiplatelet treatment also influences circulating levels of miR-126. In their study, the circulating levels of miR-126 were significantly reduced after switching from DAPT with ASA and clopidogrel to ticagrelor (50).

In line with these results, miR-126 was reported to correlate with elevated risk for MI (38). Yu et al. suggested that plasma miR-126 may serve as a future marker prognostic of major adverse cardiac events in patients after percutaneous coronary intervention (PCI) in a study conducted on 491 Han Chinese individuals who had received PCI and DAPT (51). On the contrary, Schulte et al. reported in their work that miR-126 expression did not demonstrate any significant prognostic value, neither in the overall group, nor in ACS or the stable CAD group (37). Given the diverging results, it is mandatory to perform further studies in order to validate the usefulness of miR-126 in assessment of cardiovascular death risk stratification.

Summing up the above reported results, accumulating evidence indicates that miR-126 might be used as an innovative biomarker and potential novel therapeutic target through its roles in maintaining endothelial homeostasis. However, to better evaluate the potential role of miRNA-based therapy more studies will be required to investigate the intricate interactions between this miRNA and its target genes in T2DM, CAD, and other CVD.

miR-197

miR-197, found on human chromosome 1, is among the most highly expressed miRNAs in platelets (1, 38, 52). However, its role in platelet activation is not fully defined, yet. In addition, it has been established that miR-197 might contribute to dyslipidemia in metabolic syndrome, hence leading to the progression of CVD (53).

Schulte and colleagues evaluated the prognostic aspect of serum-derived circulating miR-197 in a large population of patients with CAD ($n = 873$). Cardiovascular death was seen in 2.1% of the patient cohort over a follow-up period of 4 years and baseline levels of three miRNAs, one of them being miR-197, were more elevated in individuals with future cardiovascular death relative to event-free subjects. According to their results, miR-197 could serve as a prognostic biomarker of future cardiovascular death (37). Also Zampetaki and colleagues investigated the link between baseline miRNAs levels and incident MI in the Bruneck cohort and revealed that miR-197 negatively correlated with disease risk [multivariable hazard ratio: 0.47 (95% CI: 0.29–0.75), $P = 0.002$, and 0.56 (95% CI: 0.32–0.96), $P = 0.036$] (38). The team found that investigated miRNAs were predominantly expressed in platelets. These findings suggest that the observed loss of numerous miRNAs, including miR-197, may indicate abnormal platelet function in T2DM population (38). In another study, lower plasma levels of miR-197 were revealed in subjects with manifest T2DM (25). Whereas this study supported the concept of plasma miRNAs being abnormally regulated in T2DM, the underlying mechanism has not been exhaustively clarified. The results suggest that plasma miRNAs, including miR-197, might be a useful tool to predict both cardiovascular death and T2DM. However, these results require an independent validation in larger groups of CAD patients and prediabetes before more definitive comparisons with other standard risk factors can be made.

Human platelets harbor a diverse and complex miRNA repertoire. Beside the abovementioned three most known miRNAs, some other types count toward the most highly expressed miRNAs in human platelets and have a capacity to influence platelet function (54).

miR-191

miR-191 is located on human chromosome 3 and expressed both in platelets and endothelial cells (55, 56). It was one among 377 miRNAs profiled in a seminal study performed by Willeit et al., which showed remarkably higher levels of miR-191 in serum than plasma. Interestingly, plasma levels of platelet miR-191 were also found to be decreased on platelet inhibition with prasugrel and ASA (1).

In another study performed in 39 patients, Hsu et al. unveiled that miR-191-5, as well as miR-486-3p were markedly reduced in the sera of patients with acute myocardial infarction (AMI) (57), suggesting that they could be potential diagnostic biomarkers for ST segment elevation myocardial infarction (STEMI) (57). Also, Li et al. assessed the expression of miRNA in a cohort of AMI patients and healthy subjects to establish whether plasma levels of miR-26a, miR-191, and miR-208b could be clinically useful biomarkers of AMI. The study, which included 87 AMI patients and 87 healthy individuals, revealed that miR-191 and miR-26a were decreased in AMI relative to healthy subjects. A good diagnostic performance was found for miR-191 (AUC = 0.669; 95% CI: 0.589–0.749; $P < 0.001$) (58). On the other hand, Kakimoto et al. analyzed the possible application of miRNA quantification during postmortem examination of AMI patients. Among 55 samples of cardiac tissue that were collected and examined, miR-191 and miR-26b showed sufficient stability after death and long-lasting fixation, to be considered as candidate biomarkers in this setting (59).

In a population of T2DM patients, Dangwal et al. reported a three- to sixfold decrease in plasma levels of circulating miR-191 in diabetic versus healthy controls. miR-191 was correlated with cytokine levels and C-reactive protein ($r = 0.333$; $P = 0.009$) in T2DM subjects and indeed, pro-inflammatory stress caused higher secretion of endothelial- or platelet-derived miRNA (56).

Currently, cardiac troponins and creatinine kinase-MB are the most common biomarkers for MI. Nevertheless, these are biomarkers of myocardial necrosis, while miRNAs could provide broader information about a wider range of biological processes, potentially allowing an earlier diagnosis. It is therefore of paramount importance to promote further research to increase the current efficiency of detection methods for miRNAs (60). Although the usefulness of miR-191 appears to be modest, current data suggest probable involvement of platelet-secreted miRNAs in the plasma pool of T2DM patients (56).

miR-21

The miR-21-5p is located on human chromosome 17 (5, 61). It is highly expressed in cardiovascular cells, such as vascular smooth muscle cells (VSMCs), endothelial cells (ECs), cardiac fibroblasts (CF), and cardiomyocytes (CMC), as well as in platelets (38, 62).

In order to assess the potential application of atherosclerosis-related miRNAs (miR-361-5p, miR-21-5p, and miR-519e-5p) in the diagnosis of AMI, Wang et al. evaluated the expressions of circulating miRNAs in individuals with AMI. Plasma level of miR-21 in this cohort was significantly elevated relative to healthy volunteers without a history of CAD. Interestingly, circulating miR-21 exhibited similar trend to plasma cardiac troponin I in the early phase of AMI with both of them achieving the peak concentration at 4 h after initial time, and declining in the subsequent hours. Clinical application of miR-21 for diagnosing and monitoring AMI were also assessed. As miRNA quantitative analysis demonstrated elevated expression of miR-21 not only in patients with AMI but also in patients with stroke or pulmonary embolism, it could lack sufficient specificity to be exploited as a disease biomarker (63). On the other hand, Zhang et al. found that plasma levels of miR-21 were significantly higher in patients with AMI or angina compared with controls. They also found a significant correlation between miR-21 and clinically established markers, including cTnI, creatine kinase, and creatine kinase ($P < 0.001$) reinforcing the concept of the application of circulating miRNAs in disease diagnosis and prognosis (64, 65).

In another study conducted by Cengiz et al., miR-21 was investigated as a potential contributor to subclinical atherosclerosis among hypertensive patients. In a cohort of 28 hypertensive subjects and an equal number of healthy volunteers, plasma miR-21 expression was markedly elevated in the hypertension group *vs*. the control group. Furthermore, miR-21 levels were positively associated with both systolic and diastolic blood pressure, suggesting that it may play a role in initial stages of atherosclerosis in patients with hypertension (66).

On the contrary to cardiac disorders, in which miRNA-21 is the most abundantly expressed miRNA, in patients with T2DM, the plasma level of miR-21 was found to be reduced (5, 25). The same trend was confirmed by Olivieri et al. who carried out a study on 193 T2DM patients and 107 healthy control subjects; however, they also established that expression of miR-21-5p was higher in patients with T2DM and major cardiovascular events as compared to other T2DM patients (61). On the contrary to cardiac disorders, in which miRNA-21 is the most abundantly expressed miRNA, in patients with T2DM the plasma level of miR-21 was lower in patients with T2DM compared to controls (5, 25).

In summary, miR-21 is a promising biomarker of ischemic cardiovascular diseases, as well as T2DM. Nonetheless, further studies should be designed to disentangle the conflicting results available to date in T2DM.

miR-150

Despite the fact that this miRNA has not been associated to prognosis in T2DM patients, miR-150 is worth mentioning in this review, as it has an impact on platelet maturation and function. miR-150 is found on human chromosome 19 and it is known to be a key modulator of platelet production and activation (50, 67). It is highly expressed in leukocytes and monocytes, where it targets c-Myb, a transcription factor associated with cell proliferation, lineage commitment, and migration (34).

It was discovered that miR-150 levels were upregulated as megakaryocyte-erythrocyte progenitors (MEPs) differentiated toward the megakaryocyte lineage (68). The contribution of miR-150 to megakaryocyte differentiation was also confirmed by Barroga et al. In fact, they found that thrombopoietin increases the expression of miR-150 (69). Nevertheless, miR-150 was reported to influence not only platelet production, but also their activation. Willeit et al. revealed that more potent platelet inhibition in healthy subjects resulted in lower levels of miR-150 (1). Correlation between miR-150 level and platelet activation is further and independently supported by experimental result from our group (50). In fact, similarly to miR-126, the circulating levels of miR-150 were significantly lower after the switch from clopidogrel to ticagrelor (50). Furthermore, Yu and colleagues found that miR-150 is upregulated in platelets by apheresis (70).

The diagnosis of unstable angina (UA) based on time-specific biomarker remains a major clinical challenge. Zeller et al. performed a study aiming to assess clinical utility of miRNAs as a new tool in patients with UA. By using a three-phased profiling-replication-validation model, they found eight miRNAs which could be used clinically in the early diagnosis of UA. Among selected miRNAs, miR-186 demonstrated the greatest correlation with UA. However, the triple-miRNA combination of miR-150, miR-132, and miR-186 was shown to be of highest diagnostic accuracy indicating that a multi-miRNA approach is more reliable than single miRNAs (71). In another study conducted by Zhang et al., the level of circulating miR-486 and miR-150 in patients with AMI and their prospective role as biomarkers for AMI were investigated. The study included 65 STEMI patients, 45 non-ST-segment elevation myocardial infarction (NSTEMI), and 110 healthy subjects. According to PCR results, plasma miR-486 and miR-150 were significantly higher in all AMI (both STEMI and NSTEMI) subjects. Moreover, miR-150 was considerably overexpressed in the initial stage of AMI in patients, relative to healthy subjects ($P < 0.001$). An evident difference was reported between the levels of plasma miR-150 and miR-486 between STEMI and NSTEMI subjects, suggesting that miR-150 could be helpful to distinguish NSTEMI patients from healthy volunteers (72). Recently, also Karakas et al. evaluated the potential role of eight miRNAs (miR-19a, miR-19b, miR-132, miR-140-3p, miR-142-5p, miR-186, miR-150, miR-210) as prognostic biomarkers for CVD in a large population of 1112 CAD patients (430 ACS patients, 682 stable CAD patients). They found that the majority of miRNAs were predictive of cardiovascular death in ACS patients during a follow-up of 4 years (73). Furthermore, Goren et al. reported that miR-150 levels correlate with platelet count in heart failure (HF) patients and its expression levels were 3.5-fold lower in subjects with HF and atrial fibrillation (AF) compared to HF subjects without AF (74).

On the basis of the above reported evidence, it can be hypothesized that miR-150 levels in peripheral blood could be used to predict mortality in secondary prevention settings.

miR-155

miR-155 is located on human chromosome 21. Its platelet content is significantly reduced in diabetics (16). miR-155 is enriched in inflammatory microvesicles in patients with cardiovascular or dysmetabolic diseases. Nevertheless, detailed information on its function in cardiovascular pathophysiology is lacking, to date. Therefore, its potential role as biomarker needs further investigation (12, 75).

miR-140

So far, data on the association between miR-140 and platelet reactivity or function are limited. This miRNA is located on human chromosome 16 (76). Fejes et al. aimed to investigate the concentration of circulating platelet miR-126, miR-140, miR-223, and miR-26b that might be modified by their target mRNAs in T2DM, in a study involving 70 subjects. It was found that miR-140, miR-223, miR-126, and miR-26b are reduced both in platelets and megakaryocytes of T2DM patients, resulting in upregulation of P2RY12 and P-selectin mRNAs. This might in turn lead to abnormal platetet function (34). Karakas et al., on the other hand, suggested that miR-140-3p is a promising prognostic marker in CAD patients (73).

miR-96

Circulating levels of miR-96, located on human chromosome 7, are associated with platelet function, both in normally responsive patients and in the setting of platelet hyperreactivity (50, 77, 78). Those findings are particularly interesting, as it was reported that upregulation of miR-96 parallels hyperexpression of vesicle-associated membrane protein 8 (VAMP8)/endobrevin, a known v-SNARE involved in platelet degranulation (78). Noteworthy, VAMP8 itself is a target of miR-96 (78).

miR-98

miR-98 that is located on human chromosome X, belongs to miRNAs, which have been scarcely described and analyzed in respect to their role in platelet function, to date (79). However, Osman et al. demonstrated that miR-98 was among the miRNAs (miR-15 a, miR-98, miR-339-3 p, miR-361-3 p, miR-365, and miR-495) that are deregulated upon platelet activation ($P \leq 0.001$). These data provide valuable information on potential miRNA target pathways in platelets, even though further studies are required to precisely evaluate their actual usefulness as biomarkers of CVD (80).

FUTURE PERSPECTIVES OF miRNA

Circulating miRNAs are stable in both plasma and serum and were shown to have prognostic values for CVD (81–84). Thus far, three main pools of plasma circulating miRNAs have been verified: protein-bound, high density lipoprotein (HDL)-associated, and microvesicle (MV)/exosome-associated miRNAs (85). It has been described that among these three pools, a significant amount of plasma miRNAs is associated to microvesicles (86). However, Arroyo et al. reported that miRNA can be also found in a vesicle-free form bound to RNA-binding proteins, including nucleophosmin and Argonaute protein 2 (83, 87, 88).

Recent data demonstrate that age and gender play a role in microRNA–RNA interactions in platelets. Given that these demographic variables have a considerable impact on cardiovascular and diabetes prevalence, morbidity, and mortality, the link between age- and gender-related differences in miRNA expression could further enhance the prognostic value of miRNA. Simon et al. evaluated mRNA and miRNA levels in platelets from 84 white and 70 black healthy subjects. Out of 5,911 mapped mRNAs and 181 miRNAs that were expressed and validated in a separate cohort, 129 mRNAs and 15 miRNAs were differentially expressed by age, and 54 mRNAs and 9 miRNAs by gender. The results suggest that miRNAs modulate mRNA levels on aging and between genders, and hence these variables could be incorporated into a predictive model for platelet reactivity biomarker (89).

As reviewed above, platelets harbor large amounts of miRNAs. Consequently, they are the major source for circulating miRNAs, with a relevant regulatory potential on cardiovascular pathophysiology. Platelet-derived miRNAs could be exploited as useful biomarkers for clinical use (90). Nevertheless, several issues should be solved to enable effective application of circulating platelet-derived miRNAs as disease biomarkers in the clinical setting. In fact, several innovations are being put forward in the diagnostic methodology. Among the others, fast PCR-based techniques merit mention. In addition, the use of primers bound on multi-well plates also represents a valuable progress. Furthermore, microfluidic systems have been also developed to filter blood samples before analysis, allowing fast detection with no need for time-consuming centrifugations. These techniques can also be associated to advanced detection methods, such as microarrays. Using an alternative approach, enzyme-linked assays are being developed, to allow miRNAs measurement through direct hybridization. Among the most promising, the use of nanosensors or nanowires will be an active field of technological development over the next years. These latter, combined with microfluidic filtering devices, will allow the development of reliable and efficient, label-free detection methods (91).

Current evidence on the pathophysiological role played by miRNAs in this specific topic is still not sufficient to support their use as therapeutic targets using currently available technologies. For example, many of the miRNAs discussed in this review are downregulated in the diseased state, while current therapeutic strategies in other fields are mostly based on inhibition of abnormally elevated miRNAs. In fact, therapeutic increase of miRNAs *in vivo* is more challenging with the currently available bio-tech armamentarium. More studies should be designed with an aim to better understand the precise involvement of miRNAs in this specific pathophysiological context, to allow the identification of specific therapeutic targets.

CONCLUSION

Upon activation, platelets secrete microvesicles that carry large amounts of growth factors, as well as various proteins which might exert extracellular effects. Recent studies have indicated that PMVs may deliver platelet miRNA to a specific site in the cardiovascular system (10). The platelet-derived miRNAs that have the highest association with CVD are miR-223 and miR-126. miR-223 regulates erythrocyte membrane protein band 4.1 like 3 (*EPB41L3*) gene, known to be linked to atherosclerosis, while miR-126 regulates a gene that is strongly linked to endothelial dysfunction and atherosclerosis as vascular cell adhesion molecule 1 (VCAM-1), therefore indicating that platelet-derived miRNAs have an impact on the regulation of key genes associated with CVD (5). In our previous studies, in a cohort of T2DM patients, we found that platelet reactivity could be related to a number of clinical factors, biochemical variables, and genetic polymorphisms (92–99). Moreover, we found that genetic polymorphisms within genes related to platelet reactivity could be also a useful prognostic tool (12). Similarly, to gene polymorphisms, miRNA profiling may expose inter-individual differentiation in platelet reactivity, disease susceptibility, or response to therapy.

Results of the studies presented in this review should be interpreted with consciousness. The discrepancy of results might stem from demographic differences between populations, heterogeneity of populations, various cohort sizes and study designs. It is necessary to conduct further studies to validate the current hypotheses and closely determine the association between various miRNA and platelet reactivity, as well as their contribution to cardiovascular diseases development.

Altogether, miR-223, miR-126, miR-197, miR-24, and miR-21 were found to be the most abundant miRNAs in human platelets and PMVs and may contribute to the plasma miRNA pool (1, 43, 100). Mounting evidence suggests that platelet miRNAs could be exploited as biomarkers in inflammatory diseases, including T2DM and CVD, as they influence a broad spectrum of cell mechanisms and functions. Given the abundance of platelets in the blood and their substantial contribution to the circulating miRNA pool, these cells could serve as the main source for this class of biomarkers. Presently, few biomarkers could be applied clinically to verify subjects at higher risk of development of acute presentation of CVD. Despite use of classical cardiovascular risk factor and the development of numerous risk stratification models, a significant proportion of cardiovascular risk still eludes currently available risk stratification strategies (101). This residual risk is partly related to genetic variability, partly associated to environmental factors that are not captured by available risk models (102, 103). Hence, miRNAs could be useful epigenetic biomarkers, while able to sense both genetic and environmental risk components.

AUTHOR CONTRIBUTIONS

JP, KP, SR, and MP: substantial contribution to concept and design and critical writing or revising the intellectual content. SR and MP: edition of the manuscript and supervision of the work. CE: valuable contribution to graphic design. CI: critical revision of the article. JP, KP, SR, MP, CE, CI, ADJ and LM: collection, analysis, and interpretation of data; verification of analytical methods; and final approval of the version to be published. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fendo.2018.00074/full#supplementary-material.

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Vitamin D on Early Stages of Diabetic Kidney Disease: A Cross-sectional Study in Patients with Type 1 Diabetes Mellitus

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Context: Genetic and environmental factors are involved in the pathogenesis of type 1 diabetes mellitus (T1DM), and vitamin D (VD) deficiency appears as a candidate to risk factor for developing diabetic kidney disease (DKD).

Objective: The purpose of study was to evaluate the existence of an association between low levels of VD and the presence and degree of DKD in T1DM.

Patients and methods: We performed a cross-sectional study, between November 2014 and December 2015. Levels of 25(OH)D and albuminuria were analyzed in 37 patients with T1DM and normal glomerular filtration rate. Thirty-six subjects were evaluated as a control group.

Results: Patients with T1DM and hypovitaminosis D had higher levels of albuminuria compared to those with normal VD levels [albuminuria ($\log_{10}$) = 1.92 vs. 1.44; $p < 0.05$]. When we have separated the group of patients according to stage of DKD in patients with normo, micro, and macroalbuminuria, there are lower levels of 25(OH)D in the last when compared to the first two groups (26.7 ± 6.2, 24.8 ± 7.0, and 15.9 ± 7.6 ng/ml; $p < 0.05$, respectively). In T1DM group, we have found correlations between VD levels and both albuminuria and DKD stages ($r = -0.5$; $p < 0.01$ and $r = -0.4$; $p < 0.05$, respectively). A simple linear regression model, with albuminuria as the dependent variable and VD as an independent variable, showed $r^2 = 0.2$ and $p < 0.01$.

Conclusion: Our data suggest an association between reduced levels of VD and the presence and severity of DKD.

Keywords: type 1 diabetes mellitus, albuminuria, vitamin D, 25(OH)D, diabetic kidney disease

INTRODUCTION

Both environmental and genetics have been known as risk factors involved in the pathogenesis of type 1 diabetes mellitus (T1DM). The vitamin D (VD) deficiency appears as a candidate to risk factor for developing T1DM and diabetic kidney disease (DKD).

The VD deficiency is associated with increased urinary albumin excretion, as well as an increase in the prevalence of cardiovascular disease and mortality in patients with chronic kidney disease in the general population, which has decreased levels of this vitamin (1, 2). The great difficulty in assessing relationship between low levels of VD and DKD is the fact of renal injury itself cause reduced levels of VD. It makes difficult to establish whether VD deficiency would be a triggering environmental factor of DKD or just a consequence of this progression.

A few studies evaluating VD levels in the early stages of DKD and its association with the presence of microalbuminuria have found conflicting results (3–5). Therefore, it is important to establish the existence of a direct relationship between low levels of VD and the presence of DKD in T1DM, particularly in early cases, with normal glomerular filtration rate (GFR).

Therefore, the purpose of the present study was to evaluate the existence of an association between low VD levels with the presence and degree of DKD in T1DM with normal GFR.

MATERIALS AND METHODS

Study Design and Patients

We performed a cross-sectional study, between November 2014 and December 2015, in which levels of 25(OH)D and albuminuria were analyzed. Patients were recruited from Endocrinology Division of the Federal University of Pará, included 37 T1DM with normal GFR (≥90 ml/min/1.73 m^2) (6) and 36 controls with normal serum creatinine and without comorbidities. The control group was recruited in communities near to University Hospital. Both groups were matched by age and GFR. Exclusion criteria were pregnancy, lactation, individuals with a history of liver disease, use of VD/calcium in the last 6 months, prior concomitant history of metabolic bone diseases, hyperthyroidism or hypothyroidism, and patients. The study was approved by the University Hospital João de Barros Barreto ethics committee – protocol No. 2158/11, and it was in accordance with the standards of the National Health Council. Informed consent was obtained from all patients for being included in the study.

Clinical and Laboratorial Data

All patients had the following clinical parameters measured: weight, height, body mass index (BMI), and systolic and diastolic blood pressure. Diabetic patients (group 1) and controls (group 2) were submitted to the following laboratory tests: glycated hemoglobin (HbA1c), serum creatinine, 25(OH)D, TSH, free T4, total cholesterol and fractions, triglycerides, and fasting glucose. Albuminuria in three 24 h urine samples was measured only in T1DM. HbA1c was measured by HPLC. The levels of 25(OH)D were measured by chemiluminescence immunoassay (7) and are classified according to their levels: deficiency (<20 ng/ml), insufficiency (20.0–29.9 ng/ml), and normal (≥30.0 ng/ml). The GFR was calculated by formula CKD-EPI (8). The DKD was graded in stages by albuminuria and GFR. Albuminuria was performed by immunoturbidimetry (9), and T1DM was classified according to results in normoalbuminuria (<30 mg/g creatinine), microalbuminuria (≥30 mg/g creatinine and <300 mg/g creatinine), and macroalbuminuria (≥300 mg/g creatinine).

Statistical Analysis

Categorical variables were described as frequency (percentage), numeric variables with normal distribution were described as mean (SD), and the other as median (minimum–maximum). Chi-square and Fisher tests were used to compare categorical variables. The Student's *t*-test and Mann–Whitney test were used to compare two groups of numerical variables with and without normal distribution, respectively. To establish correlations between variables, Pearson and Spearman tests were used. The ANOVA test compared more than two groups of numerical variables with normal distribution, and the Kruskal–Wallis test was used to compare more than two groups of numerical variables without normal distribution.

In regard to DKD, the stage, normoalbuminuria, microalbuminuria, and macroalbuminuria, was given as DKD indexes the numerals 0, 1, and 2, respectively. Similarly, in regard to VD levels, the status, normal, insufficiency, and deficiency, was given as VD indexes the numerals 0, 1, and 2, respectively. For clarification, the index was used for statistical analysis.

Additionally, albuminuria values were converted to log base 10 ($\log_{10}$) to better analyze the data. A simple linear regression model was analyzed using albuminuria as dependent variable and VD as independent variable. We have also created a regression model (backward stepwise) with albuminuria as dependent variable and age, sex, BMI, SBP, DBP, 25(OH)D, HbA1c, and duration of T1DM to determine the important of those variables as independent predictors. After that, another multiple linear regression model was used to verify if a combination of the variables selected from the backward stepwise model could increase the predictive value to albuminuria when compared to the simple linear regression model using only VD levels. Interferences were represented by hypothesis test with a significance level of 0.05 bilaterally. All information was stored and processed using the software Statistical Package for Social Sciences (SPSS) 21.0 (IBM).

RESULTS

The clinical and laboratorial characteristics of T1DM and controls are shown in **Tables 1** and **2**, respectively.

There were no differences between T1DM and controls in the number of patients at different stages according to VD levels (**Figure 1**). The prevalence of hypovitaminosis D among controls was quite high (78%), and there was no difference in relation to T1DM, whose prevalence was 73%. For DKD stage, it was found that 8 patients (21.6%) had normoalbuminuria, 25 patients (67.6%) had microalbuminuria, and 4 patients (10.8%) had macroalbuminuria. The VD levels in different stages of DKD are shown in **Table 3**.

Patients with T1DM and hypovitaminosis D had higher levels of albuminuria compared to those with normal VD levels [albuminuria ($\log_{10}$) = 1.92 vs. 1.44; $p < 0.05$].

A correlation was found between VD and albuminuria (transformed into $\log_{10}$) in T1DM (**Figure 2**). In addition, we have

TABLE 1 | Clinical characteristics of T1DM and controls.

	Sex (M/F)	Age (years)	DT1DM (years)	PAS (mmHg)	PAD (mmHg)	IMC (kg/m²)
T1DM (*N* = 37)	13/24	28.6 ± 8.1	13.2 ± 6.6	120.3 ± 13.6	76.1 ± 9.6	24.2 ± 3.9
Controls (*N* = 36)	19/17	25.2 ± 3.0	–	119.1 ± 12.3	74.0 ± 7.9	26.7 ± 5.8
p	NS	NS	–	NS	NS	<0.05

DT1DM, duration of diabetes mellitus; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; NS, not significant.

TABLE 2 | Laboratory characteristics of T1DM and controls.

	HbA1c (%)	Vitamin D (ng/ml)	GFR (ml/min/1.73 m²)	Microalbuminuria (mg/24 h)	Microalbuminuria (log_{10} mg/24 h)
T1DM (*N* = 37)	10.3 ± 3.2	24.2 ± 7.4	95 ± 20	236.2 ± 690.7	1.78 ± 0.36
Controls (*N* = 36)	5.2 ± 0.3	25.8 ± 11.2	92 ± 11	–	–
p	<0.05	NS	NS	–	–

log_{10}, transformation of microalbuminuria value to log_{10}; GFR, glomerular filtration rate; HbA1c, glycated hemoglobin; NS, not significant.

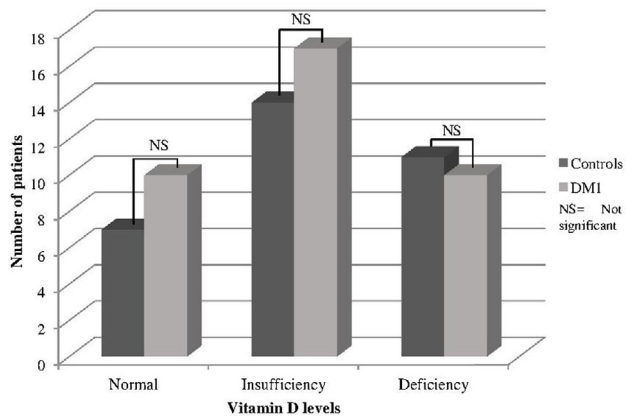

FIGURE 1 | Status according to VD levels in T1DM and controls.

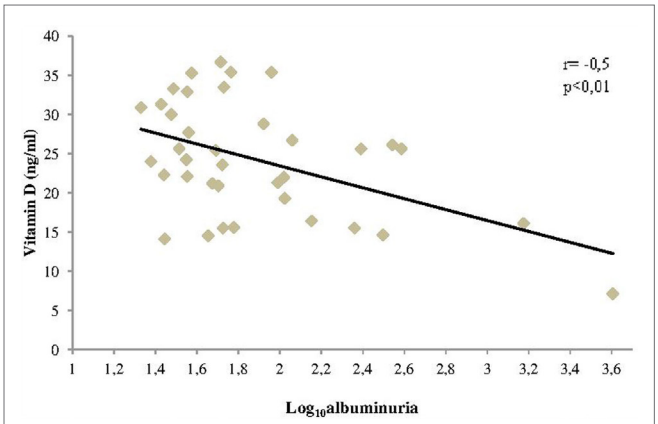

FIGURE 2 | Correlation between vitamin D levels and albuminuria in patients with T1DM.

TABLE 3 | Vitamin D levels and DKD stages in patients with T1DM.

	DKD stages		
	Normoalbuminuria (mg/24 h)	Microalbuminuria (mg/24 h)	Macroalbuminuria (mg/24 h)
Vitamin D (ng/ml)	26.7 ± 6.2	24.8 ± 7.0	15.9 ± 7.6*

**p < 0.05 vs. normoalbuminuria and microalbuminuria.*

found a progressive decrease of VD levels as the stage of DKD worsened (**Figure 3**). In addition, albuminuria levels according to the status of VD levels had similar behavior (**Figure 4**).

Our simple linear regression model, with albuminuria as the dependent variable and VD as an independent variable, showed a $r^2 = 0.2$ and $p < 0.01$. The β1 coefficient was equal to −43. This suggests that every increase of 1 ng/ml of VD could result in a reduction of 43 mg/g in albuminuria.

Our backward stepwise regression model has found that VD, HbA1c, and duration of T1DM were independent predictors of albuminuria. When we have included those three variables in a multiple linear regression model, we have found an increase in predictive value to albuminuria when compared to 25(OH)D alone ($r^2 = 0.34$ and $p < 0.01$).

DISCUSSION

Our data suggest an association between reduced levels of VD and the presence and severity of DKD in T1DM. There are few studies (3–5) assessing VD levels in the early stages of DKD and its association with the presence of microalbuminuria with conflicting results. Joergensen et al. (4) in 2011, evaluated VD as a predictor for progression of normoalbuminuria to microalbuminuria in T1DM. To this end, they collected VD before the development of microalbuminuria, and these patients were followed for about 26 years. In this period, 81% of patients developed microalbuminuria, and this was not associated with reduced levels of VD. Differently, Verrotti et al. (3) have compared VD levels and urinary albumin excretion in 22 patients with T1DM and normoalbuminuria, 24 T1DM and microalbuminuria, and 24 controls. Their findings showed lower levels of 25(OH)D in patients with microalbuminuria when compared to other groups. Additionally, de Boer et al. have tested associations

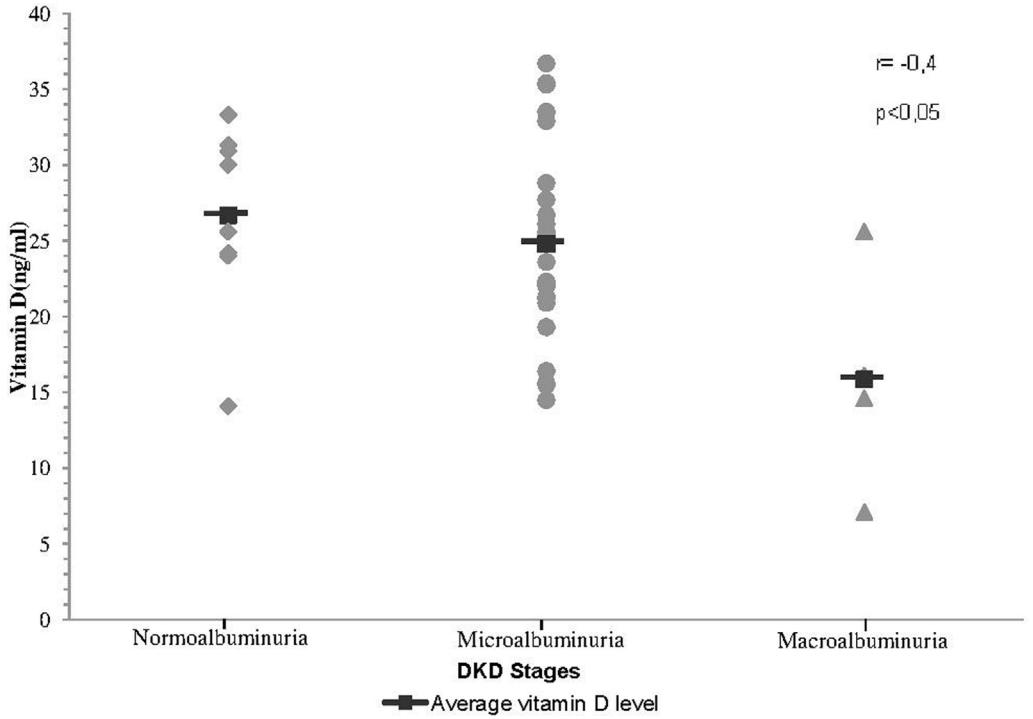

FIGURE 3 | **Correlation between VD levels and stages of DKD**.

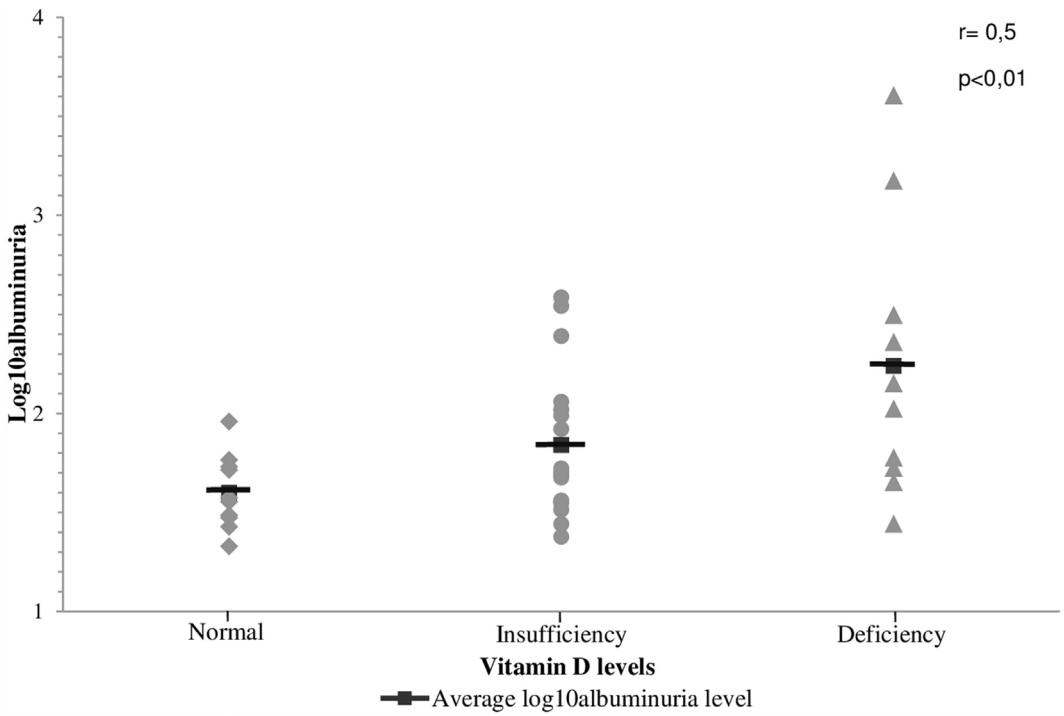

FIGURE 4 | **Correlation between the levels of albuminuria and the status of vitamin D levels in patients with T1DM**.

between circulating VD metabolites and microalbuminuria in T1DM. They have found that low plasma concentrations of VD (below 20 ng/ml) were associated with an increased risk of microalbuminuria in 65% of patients. Our findings reinforce the data described by de Boer et al. (5). Although there was no difference between the levels of 25(OH)D between T1DM with normo and microalbuminuria, we have demonstrated a good correlation between the reduction in VD levels and evolution of DKD stages.

It has been reported involvement of VD deficiency in the development of diabetes (10). However, it is unclear whether VD deficiency could be an environmental factor involved in the pathophysiology and progression of DKD. The VD receptors (VDRs) are present in the kidney (11). Zhang et al. (12) in 2008 has demonstrated that inactivation of the VDR results in development of more severe DKD in rats, suggesting a renoprotective role of VD against renal injury by regulating the renin–angiotensin system and other genes. In their study, diabetic rats were separated according to presence or not of VDR. After 5 weeks, both groups developed abnormal albuminuria; however, this was more precocious and significant in the group of animals with absent VDR. In addition, WuWong et al. (13) in 2013 demonstrated for the first time that calcitriol exhibits a consistent effect on regulating VDR target gene expression, and calcidiol appears to have differential effects on the expression of different genes. It is not well studied whether the prehormones have effects similar to VDR agonists in providing cardiovascular benefits and/or improving survival in CKD. The VITAL study (14) has showed that treatment for 24 weeks with 2 μg of paricalcitol (VD analog) in patients with type 2 diabetes and DKD results in a reduction on excretion of residual albuminuria. In contrast, a reduction has not been demonstrated in individuals using 1 μg daily, as well as those on placebo. Additionally, it is unclear if an increase of VD levels to >30 ng/ml during a long time could help to elucidate whether VD have possible therapeutic effect on DKD in early stages. Therefore, there are no studies indicating the optimal level of VD supplementation.

A possible role of VD in DKD is the loss of VD carrier proteins (BPD). Thrailkill et al. (15) have analyzed the renal loss of BPD in diabetic patients and have found an increase in the urinary loss of this protein, with a higher increase in patients with abnormal albuminuria, suggesting that this mechanism could collaborate with hypovitaminosis D. Therefore, the question whether the urinary loss of this protein would be a risk marker of renal injury or a causal factor could be raised. Our regression data points to a VD direct action mechanism on urinary albumin excretion independently of diabetic glycemic control.

An interesting study design that could evaluate the role of VD as an environmental factor of DKD could be cohort study in T1DM with normoalbuminuria with evaluation of VD levels to observe the development of DKD. However, this would be an unethical study, due to the need to replace VD when deficiency is detected. The closest way to answer this question would be through the treatment of T1DM with newly diagnosed microalbuminuria, even in the absence of VD deficiency, in an attempt to maintain VD levels above 30 ng/ml and follow the progression of DKD. If this hypothesis is confirmed, a major step would be taken with the use of VD in these cases.

In our study, the prevalence of VD deficiency in T1DM was not different from controls. Pozzilli et al. (16) have demonstrated high VD deficiency rates in patients with newly diagnosed T1DM when compared to controls on a sunny town of Italy in the Lazio region. At the same time, Feng et al. (17) in 2015 conducted a meta-analysis reviewing 13 articles with a total sample of 3494 participants (1790 healthy controls and 1704 with T1DM) and have confirmed the previous data. In this meta-analysis, 25(OH)D levels in T1DM were 2.61 ng/ml lower than controls. Our control group have presented higher BMI when compared to T1DM. It has been reported that excess weight is associated with VD deficiency (18), so it could justify in part the fact that we have found no differences in VD levels between the two groups.

Currently, we have no large studies to determine the prevalence of VD deficiency in Brazil. Data from the city of Recife indicate low levels of VD in postmenopausal women (43%) (10), while Linhares et al. (19) evaluating 226 children of the same population and geographic region have found no VD deficiency. In contrast, in a study with 102 holmes for the elderly in Porto Alegre, where the weather is less sunny, the prevalence of VD deficiency was considerably higher (87.5%) (20). Our region is warm, humid, and sunny; however, our population has the habit to protect yourself from the sun. These factors may have influenced our results and led to a high prevalence of VD deficiency in controls.

Among the weaknesses of our study is the low number of patients with normoalbuminuria when compared to those with microalbuminuria. This fact, associated with the great variability of albuminuria in T1DM, could have been responsible for no differences between VD levels in these two groups. The number of patients with macroalbuminuria also need to be increased. But, the last problem is more difficult to resolve because this kind of patients in general are not in early stages of DKD and do not have normal GFR.

The clinical applicability of our study would be to establish an association between VD levels and DKD in early stages, but the number of patients must be increased. Prospective studies are necessary to establish whether the replacement or supplementation of VD could be useful in the treatment of diabetes kidney disease in T1DM.

STATEMENT OF HUMAN AND ANIMAL RIGHTS

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5).

STATEMENT OF INFORMED CONSENT

Informed consent was obtained from all patients for being included in the study.

AUTHOR CONTRIBUTIONS

RL collected data, and wrote and reviewed the manuscript. FM, FR, AO, AP, JN, CC, DS, MS, NQ, ML, EY, and KF have contributed by creating the database and contacting patients. JF wrote, edited the final version, and was responsible for submitting the manuscript. All the authors read and approved the final manuscript and agreed to its submission.

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ORIGINAL RESEARCH
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Progestin and AdipoQ Receptor 3 Upregulates Fibronectin and Intercellular Adhesion Molecule-1 in Glomerular Mesangial Cells *via* Activating NF-κB Signaling Pathway Under High Glucose Conditions

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Background: Progestin and adipoQ receptor 3 (PAQR3), is a Golgi-anchored membrane protein containing seven transmembrane helices. It has been demonstrated that PAQR3 mediates insulin resistance, glucose and lipid metabolism, and inflammation. In addition, kidney inflammatory fibrosis is an important pathological feature of diabetic nephropathy (DN). Therefore, we aimed to investigate the role of PAQR3 in diabetic kidney fibrosis as well as inflammation in DN.

Object: The effect of PAQR3 on NF-κB signaling pathway, expressions of fibronectin (FN) and intercellular adhesion molecule-1 (ICAM-1) in glomerular mesangial cells (GMCs) cultured by high glucose (HG) were examined.

Method: Diabetic mouse and rat models were induced by streptozotocin (STZ). GMCs were treated with HG and transfected with PAQR3 plasmids or small-interfering RNA targeting PAQR3 or NF-κB. The protein levels of FN and ICAM-1 were examined by Western blotting, and the transcriptional activity and DNA binding activity of NF-κB were measured by dual luciferase reporter assay and electrophoretic mobility shift assay (EMSA). The interaction between PAQR3 and IKKβ (inhibitor of nuclear factor κB kinase β) was analyzed by co-immunoprecipitation.

Results: PAQR3 was increased in both STZ-induced diabetic models and HG-treated GMCs. PAQR3 overexpression further increased HG-induced FN and ICAM-1 upregulation. In contrast, silencing of PAQR3 suppressed the expressions of FN and ICAM-1. PAQR3 overexpression promoted the nuclear accumulation, DNA binding activity, and transcriptional activity of NF-κB. Mechanically, PAQR3 directly interacted with IKKβ. The upregulation effect of PAQR3 overexpression on the expressions of FN and ICAM-1 was abolished by the treatment of NF-κB siRNA or PDTC (ammonium pyrrolidinedithiocarbamate) in HG-treated GMCs.

Conclusion: PAQR3 promotes the expressions of FN and ICAM-1 *via* activating NF-κB signaling pathway. Mechanistically, PAQR3 activates NF-κB signaling pathway to mediate kidney inflammatory fibrosis through direct interaction with IKKβ in DN.

Keywords: progestin and adipoQ receptor 3, diabetic nephropathy, inflammatory fibrosis, NF-κB, inhibitor of nuclear factor κB kinase β

INTRODUCTION

Diabetic nephropathy (DN), also known as glomerulosclerosis, is believed to be a common chronic microvascular complication of diabetes, and the most prevalent cause of middle-late renal fibrosis (1–3). The main pathological characteristic of DN is glomerular sclerosis resulting from microvascular pathological changes induced by diabetes. It is a leading cause of morbidity and mortality in patients with DN (4). Glomerular mesangial cells (GMCs), the intrinsic cells in glomeruli, play crucial roles in renal physiological functions and pathological changes (5–7). The accumulated extracellular matrix (ECM) components (such as fibronectin, FN) and inflammatory mediators (such as cell adhesion molecules, ICAM-1) in GMCs are involved in glomerular basement membrane thickening and glomerular fibrosis (3, 8, 9).

It is well documented that glycolipid metabolism disorders (10), non-enzymatic glycation of proteins (11), oxidative stress and cytokine secretion (12), polyol pathway (13, 14), and MAPK pathway (15) are all involved in the development and progression of diabetic renal fibrosis. Nevertheless, there are certain other mechanisms that may yet be investigated and defined. In recent years, lots of evidence highlighted the importance of inflammation in diabetic renal fibrosis, which attains our great concern (16–20). In diabetic kidneys, the activated NF-κB signaling pathway promotes the excessive expression of inflammatory mediators, which results in continuous or amplifying inflammatory responses and the secretion of ECM, eventually causing diabetic renal fibrosis. Therefore, it is of great importance to explore the mechanism of regulating inflammation in diabetic renal fibrosis.

Progestin and adipoQ receptor 3 is localized at the Golgi apparatus with seven-transmembrane helices. PAQR3 is also known as the Raf kinase trapping to Golgi, because it can function as a spatial regulator of Raf-1 kinase by sequestrating Raf-1 to the Golgi (21, 22). The expression of PAQR3 is different in various tissues of mice and humans, with higher level in skin, liver, kidney, and testicular tissue. Previous studies confirmed that PAQR3, a new tumor suppression gene, may regulate inflammation (23, 24). Numbers of evidence indicated that PAQR3-deficient mice are resistant to high-fat-diet (HFD)-induced obesity and hepatic steatosis, accompanied by improvement of insulin resistance and insulin signaling, which suggests that PAQR3 plays a vital role in the regulation of glycolipid metabolism (25–28).

Considering that DN is characterized by renal inflammatory fibrosis and PAQR3 regulates insulin resistance, glycolipid metabolism, and inflammatory response, we are highly concerned whether PAQR3 can regulate diabetic renal inflammatory fibrosis, eventually contributing to DN. Hereby, this study was aimed to investigate the effect and the underlying mechanism of PAQR3 on NF-κB signaling pathway and expressions of FN and ICAM-1 in HG-treated GMCs.

MATERIALS AND METHODS

Reagents and Antibodies

D-Glucose was purchased from AMRESCO (Solon, OH, USA). Bovine serum albumin (BSA, Fraction V) was purchased from Mbchem (Shanghai, China). Penicillin and streptomycin were purchased from Life Technologies (Grand Island, New York, NY, USA). PDTC was purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA). Antibodies against FN and ICAM-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against NF-κB, PAQR3, and LaminB1 were purchased from Abcam (Cambridge, MA, USA). Antibody against α-tubulin was purchased from Sigma (St. Louis, MO, USA). Antibody against IKKβ was purchased from Cell Signaling Technology (Boston, MA, USA). Rabbit IgG was purchased from Beyotime (Haimen, China). Alexa Fluor 488 goat anti-rabbit IgG was purchased from Rockford (IL, USA).

Cell Culture

Primary GMCs were isolated from glomeruli of Sprague–Dawley (SD) rats (about 150 g) and identified with specific assay (29). Briefly, the cortex fragments were cut into 1–2 mm pieces and sieved by specific mesh sizes (175, 147, and final 74 μm) mechanically. In the end, the filterable fragments were collected and digested with 0.1% collagenase IV in serum-free DMEM (Gibco, Carlsbad, CA, USA) for 20–30 min at 37°C. Then the digested matters were seeded in flasks with growth media (FBS, 20%; insulin, 0.66 U/mL; L-glutamine, 2 mM; 100 U/mL penicillin, and 100 U/mL streptomycin in DMEM), and incubated at 37°C under an atmosphere of 5% CO_2. The GMCs were used at passages between the 5th and 12th and cultured in normal glucose DMEM (NG, 5.6 mM) with 10% FBS. GMCs were treated with serum-free DMEM for 12 h at 80% confluent and then treated with high glucose (HG, 30 mM) or other stimuli.

Western Blot Assay

Western blot assay was performed with the standard protocol as previously described (29, 30) to detect the related proteins. GMCs or kidney tissues were harvested and lysed in RIPA lysis buffer [50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS] with protease inhibitor cocktail, phosphatase inhibitor A and B for 30 min. After centrifuged at 12,000 *g* for 15 min at 4°C, total proteins were collected. Besides the nuclear proteins and cytoplasmic proteins could harvest by a commercially available assay kit purchased from Active Motif

(Carlsbad, CA, USA). Protein concentration was determined using a BCA™ Protein Assay Kit (Pierce, USA) following the protocol of the manufacturer. An equal amount of collective proteins from cells or tissues were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then the proteins were transferred to PVDF membrane (Millipore, CA, USA) and blocked with 5% skim milk at room temperature for 1 h before being incubated with primary antibodies overnight at 4°C. After incubation, the membrane was washed with 0.1% Tween-20/TBS (TBST) and incubated with corresponding HRP-conjugated secondary antibodies (anti-rabbit IgG, anti-mouse IgG, or anti-goat IgG 1:10,000) at room temperature for 1 h. The signals were visualized with ImageQuant LAS 4000 mini obtained from GE healthcare (Waukesha, WI, USA) and then analyzed using the Quantity One Protein Analysis Software purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Immunofluorescence Staining

Glomerular mesangial cells were seeded on the glass cover slips. After transfection with plasmids or siRNA targeting PAQR3 for 24 h, the cells were washed with cold phosphate-buffered saline, fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% TritonX-100 for 10 min, blocked with 10% goat serum for 1 h, and then incubated with primary antibodies overnight at 4°C. After washing, the cells were incubated with fluorescent secondary antibody in the darkroom at room temperature for 1 h. The nuclei were labeled with 40, 6-diamidino-2-phenylindole (DAPI, Sigma, USA) for 10 min. Finally, the images were captured using a laser scanning confocal fluorescence microscope (LSM510, Carl Zeiss, Germany).

Cell Transfection of Plasmids and Small-Interfering RNAs

Transfection of His-tagged PAQR3 plasmids was performed according to the manufacturer's instruction using LTX reagent and PLUS™ reagent (Molecular Probes, Eugene, OR, USA). GMCs were cultured for 24 h prior to transfection, and then transfected with 2 μg of plasmids for 48 h. After further treatment, the cells were harvested for Western blot analysis.

Three pairs of small-interfering RNAs of PAQR3 or NF-κB were purchased from Gene Pharma (Shanghai, China). The most valid oligonucleotides of PAQR3 were si835 and their sequences were as follows: sense: 5′-GAUUGUGAUGUACGUGAUUTT-3′, antisense: 5′-AAUCACGUACAUCACAAUCTT-3′; the sequences of the most valid oligonucleotides of NF-κB were as follows: sense: 5′-GCUCGUGAGGGAUCUGCUATT-3′, antisense: 5′-UAGC AGAUCCCUCACGAGCTT-3′. GMCs were transfected with PAQR3 siRNA or NF-κB siRNA using RNAiMAX transfection reagent (Life Technologies, Grand Island, New York, NY, USA) according to the manufacturer's protocol and then incubated for 48 h. After further treatment, the cells were harvested for Western blot analysis.

Dual Luciferase Reporter Assay

Glomerular mesangial cells were seeded in 96-well plate and cotransfected with 0.2 μg pNF-κB-Luc (Beyotime, Haimen, China) and 0.02 μg pRL-TK (Promega, Madison, WI, USA) in the presence or absence of 0.05 μg of Penter-his-PAQR3. After treatment with HG, cells were harvested to analyze the luciferase activity by the Dual-Glo® Luciferase Assay System kit (Promega, Madison, WI, USA). Luciferase activity was normalized to the renilla luciferase activity.

Electrophoretic Mobility Shift Assay

Nuclear proteins were extracted by a nuclear extract kit, and the DNA binding activity of NF-κB was measured by EMSA (Thermo Fisher Scientific, Rockford, IL, USA) according to manufacturer's instruction. The sequence of the biotin-labeled oligonucleotide probes for NF-κB was as follows: 5′-AGTTGAGGGGACTTTC CCAGG-3′. 6 μg of nuclear proteins were incubated with the mixtures containing 50 ng/mL poly (dIdC), 0.05% Nonidet P-40, 5 mM $MgCl_2$, and 2.5% glycerol for 10 min. The mixtures were incubated with NF-κB probes for another 20 min at room temperature, separated by 6% non-denaturing PAGE, transferred to nylon membrane for DNA cross-links for 15 min, and then blocked for 1 h. After washed, the membrane was bound with horseradish peroxidase-conjugated streptavidin antibodies (1:300) for 15 min, last visualized and quantified with enhanced chemiluminescence by ImageQuant LAS 4000 mini (GE Healthcare, USA).

Immunoprecipitation

After treatment with HG, GMCs were harvested and lysed with immunoprecipitation buffer on ice for 30 min. After centrifuged at 12,000 *g* for 10 min at 4°C, the supernatant was collected. 300 μg of proteins were incubated with 2 μg of test antibody or rabbit IgG overnight at 4°C with shaking. 20 μL of protein agarose A/G beads was added to that mixture for further incubation. After shaking for 2 h at 4°C, the beads were washed three times with immune-precipitation buffer 1, 2, and 3 successively. 15 μL of SDS loading buffer was added to that beads and boiled twice for 5 min. At last, immunoprecipitate was followed by Western blotting with respective antibodies.

Animal Model

Animal experiments were carried out as previously described (30). Male C57/BL6 mice ($n = 14$) and SD rats ($n = 14$) were obtained from the Laboratory Animal Center, Sun Yat-sen University, Guangzhou, China. The experimental diabetic models ($n = 7$) were induced by intraperitoneal injection of freshly prepared STZ (40 mg/kg) in citrate buffer once a day for five continuous days. Control group ($n = 7$) were injected with an equal volume of citrate buffer (pH 4.5). Diabetic mice or rats with fasting blood glucose levels more than 11.1 or 16.7 mM were considered as experimental diabetic rodents, respectively. Diabetic rodents were fed with HFD for 8 weeks before their kidneys were collected for Western blot analysis. All experimental procedures were carried out in accordance with the China Animal Welfare Legislation, and approved by the Ethics Committee on the Care and Use of Laboratory Animals of Sun Yat-sen University.

Statistical Analysis

All experiments were repeated at least three times. The data were assessed by the Graphpad Prism 5.0 software and values were expressed as mean ± SD. Data were analyzed by Unpaired

Student's *t*-test for comparison between two groups, and by one-way ANOVA with *post hoc* multiple comparisons for multiple comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

The Expression of PAQR3 Was Upregulated in Kidneys of Diabetic Animals or in HG-Induced GMCs

To determine the changes of PAQR3 expression in the kidneys of diabetic animals, we first examined PAQR3 protein level in STZ-induced diabetic kidneys by Western blot assay. Compared with the mice or rats in control group, PAQR3 expression was upregulated in diabetic rodents (**Figures 1A,B**) and up to 4.75- and 4.5-fold, respectively. Furthermore, HG stimulation enhanced PAQR3 protein expression in a time-dependent manner (**Figure 1C**). Therefore, these results indicated that the upregulation of PAQR3 may be involved in diabetic renal fibrosis.

Overexpression of PAQR3 Induced the Expressions of FN, ICAM-1 in HG-Cultured GMCs

In the aforementioned experiments, we observed the upregulation of PAQR3 in diabetic kidneys and HG-treated GMCs. GMCs were transfected with plasmid expressing his-PAQR3. Our data showed that HG stimulation significantly increased the expressions of FN and ICAM-1, which was further increased by PAQR3 overexpression (**Figures 2A,B**). These results suggested that the upregulation of PAQR3 by HG promotes renal inflammatory fibrosis.

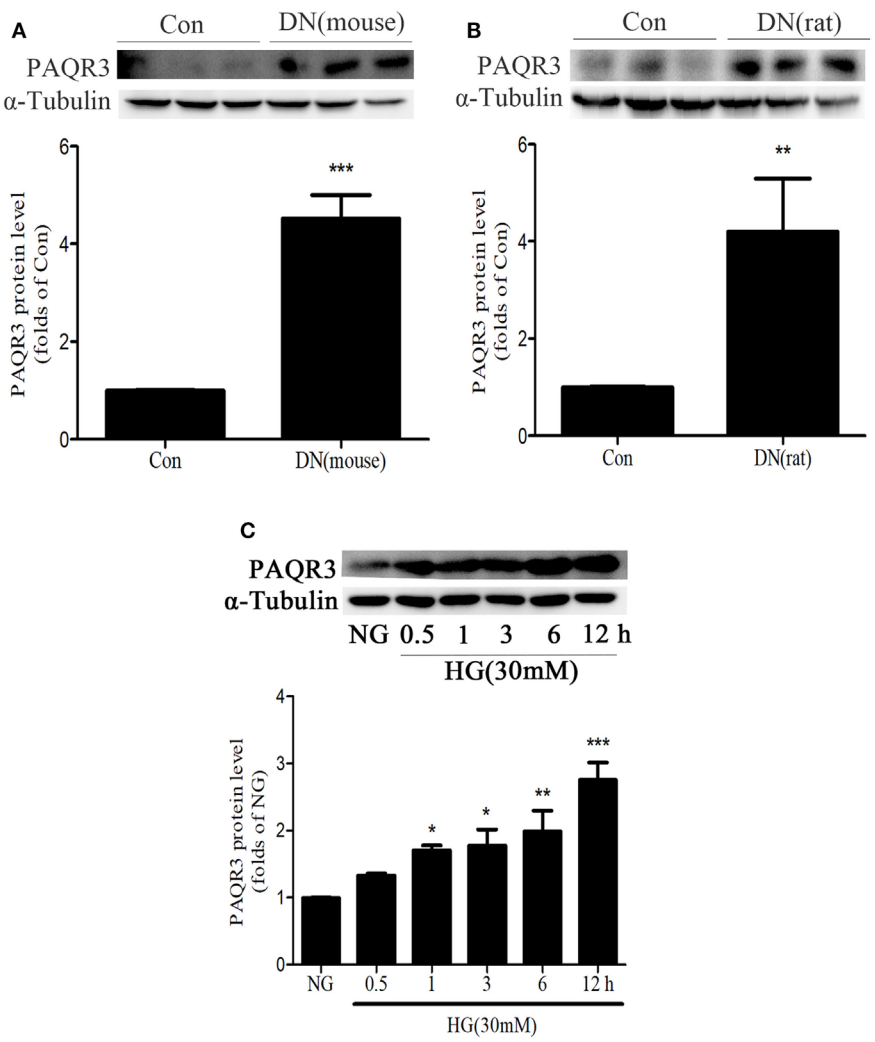

FIGURE 1 | The expression of progestin and adipoQ receptor 3 (PAQR3) was upregulated in kidneys of diabetic animals or in high glucose (HG)-induced glomerular mesangial cells (GMCs). The expression of PAQR3 was assessed by Western blot in Con or the STZ-induced diabetic mice **(A)** and rats **(B)** kidneys. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Con. After treatment of GMCs with 30 mM HG, the PAQR3 protein level was raised in a time-dependent manner (0, 0.5, 1, 3, 6, and 12 h) **(C)**. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. NG. Independent experiments were performed at least three times with similar results.

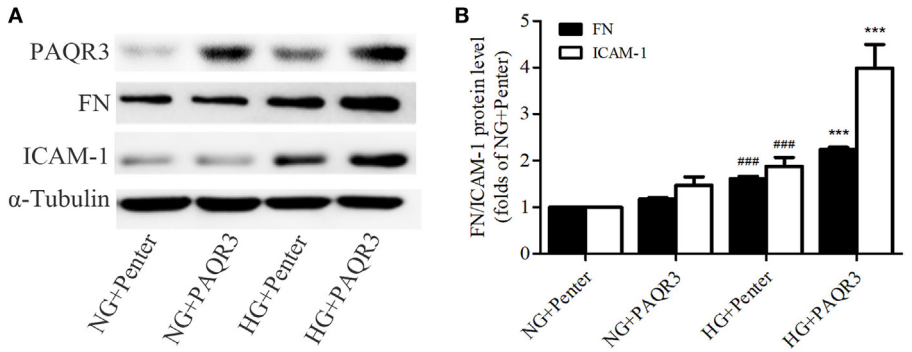

FIGURE 2 | Overexpression of progestin and adipoQ receptor 3 (PAQR3) induced the expressions of fibronectin (FN), intercellular adhesion molecule-1 (ICAM-1) in high glucose (HG)-cultured glomerular mesangial cells (GMCs). GMCs were transfected with 2 μg of Penter vector or his-PAQR3, respectively. After treatment with HG for 24 h, total proteins were extracted to measure expressions of PAQR3, FN, and ICAM-1 **(A,B)**. ###$P < 0.001$ vs. NG + Penter, ***$P < 0.001$ vs. HG + Penter. Independent experiments were performed at least three times with similar results.

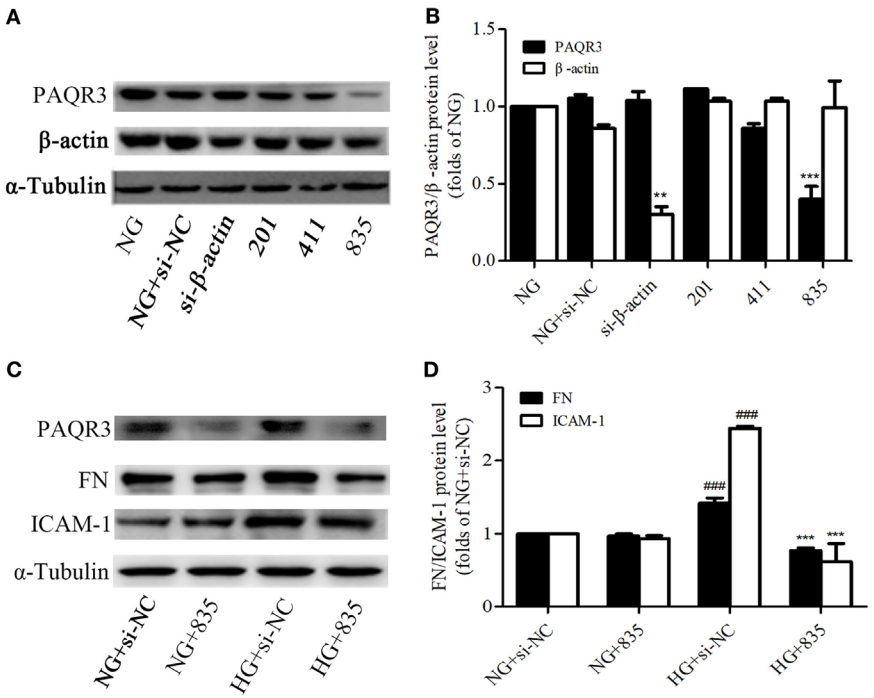

FIGURE 3 | Progestin and adipoQ receptor 3 (PAQR3) depletion reduced the high glucose (HG)-induced fibronectin (FN) and intercellular adhesion molecule-1 (ICAM-1) expressions. Glomerular mesangial cells were transfected with negative control and three pairs of siRNA oligonucleotides targeting PAQR3 for 72 h, and then total proteins were harvested and subjected to Western blot assay. NC is a short form of negative control, and the depletion of β-actin protein acts as a positive control, which is used to evaluate the efficiency of PAQR3 depletion **(A,B)**. **$P < 0.01$ vs. NG, ***$P < 0.001$ vs. NG. PAQR3 depletion inhibited the upregulation of FN and ICAM-1 under HG conditions **(C,D)**. ###$P < 0.001$ vs. NG + si-NC, ***$P < 0.001$ vs. HG + si-NC. Independent experiments were performed at least three times with similar results.

PAQR3 Depletion Reduced the HG-Induced FN and ICAM-1 Expressions

Besides of studying the effect of exogenous PAQR3, we determined the effect of endogenous PAQR3 on FN and ICAM-1 expressions in GMCs under HG conditions as well. PAQR3 was knocked down by transfection of PAQR3 siRNA in GMCs. As results shown in **Figures 3A,B**, si835 could significantly reduce the PAQR3 protein level in GMCs. The upregulation of FN and ICAM-1 by HG was strikingly reversed after PAQR3 silencing (**Figures 3C,D**). These findings further suggested that PAQR3 may play an important role in DN by regulating FN and ICAM-1 expressions.

HG Stimulation Enhanced NF-κB Nuclear Translocation in GMCs

NF-κB is an important nuclear transcription factor in mediating the inflammation as well as fibrosis lesions. Under HG conditions, NF-κB signaling pathway is activated in GMCs as demonstrated by increasing p65 nuclear translocation, promoting the transcription of downstream target genes. After treatment with 30 mM HG, the nuclear accumulation of p65 was increased. It reached a maximum level at 0.5 h and then decreased at 1 h (**Figures 4A–C**).

Upregulation of PAQR3 Further Increased the NF-κB Nuclear Translocation, DNA Binding Activity, and Transcriptional Activity in GMCs

To explore the effect of PAQR3 on NF-κB signaling pathway in GMCs with HG stimulation, PAQR3 was overexpressed and NF-κB nuclear translocation, transcriptional activity, and DNA binding activity were detected. We found that the nuclear accumulation of NF-κB was increased under HG conditions, which was further augmented by PAQR3 overexpression (**Figures 5A,B**).

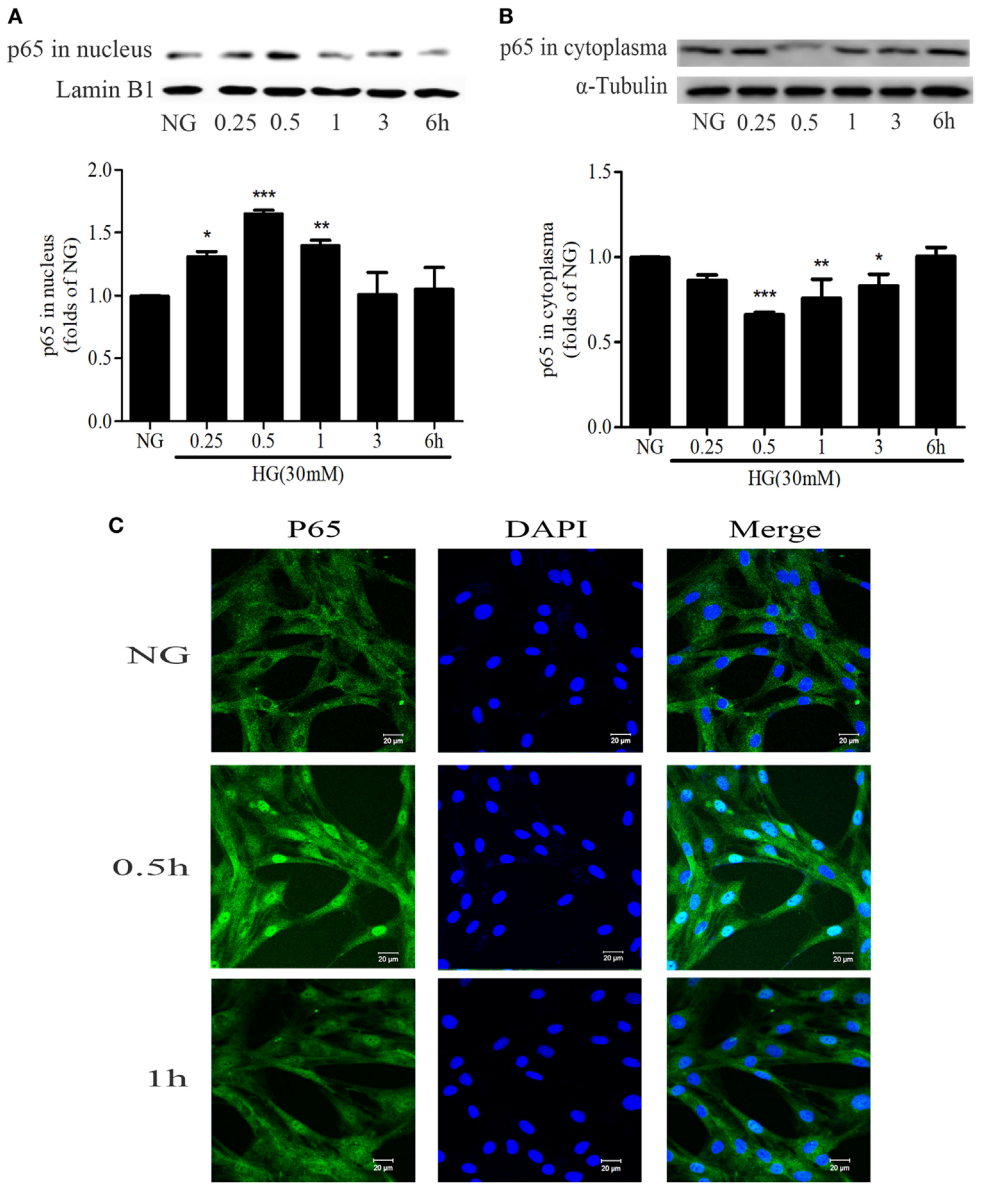

FIGURE 4 | High glucose (HG) stimulation enhanced NF-κB nuclear translocation in glomerular mesangial cells. HG-induced nuclear accumulation of p65 reached a maximum level at 0.5 h, and then decreased at 1 h **(A,B)**. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. NG. Immunofluorescent staining showed the subcellular distribution of p65 in nuclei and cytoplasm at the indicated time points (0, 0.5, and 1 h) under HG conditions **(C)**. Blue and green stains indicate nuclei and p65, respectively. Bar: 20 μm. Independent experiments were performed at least three times with similar results.

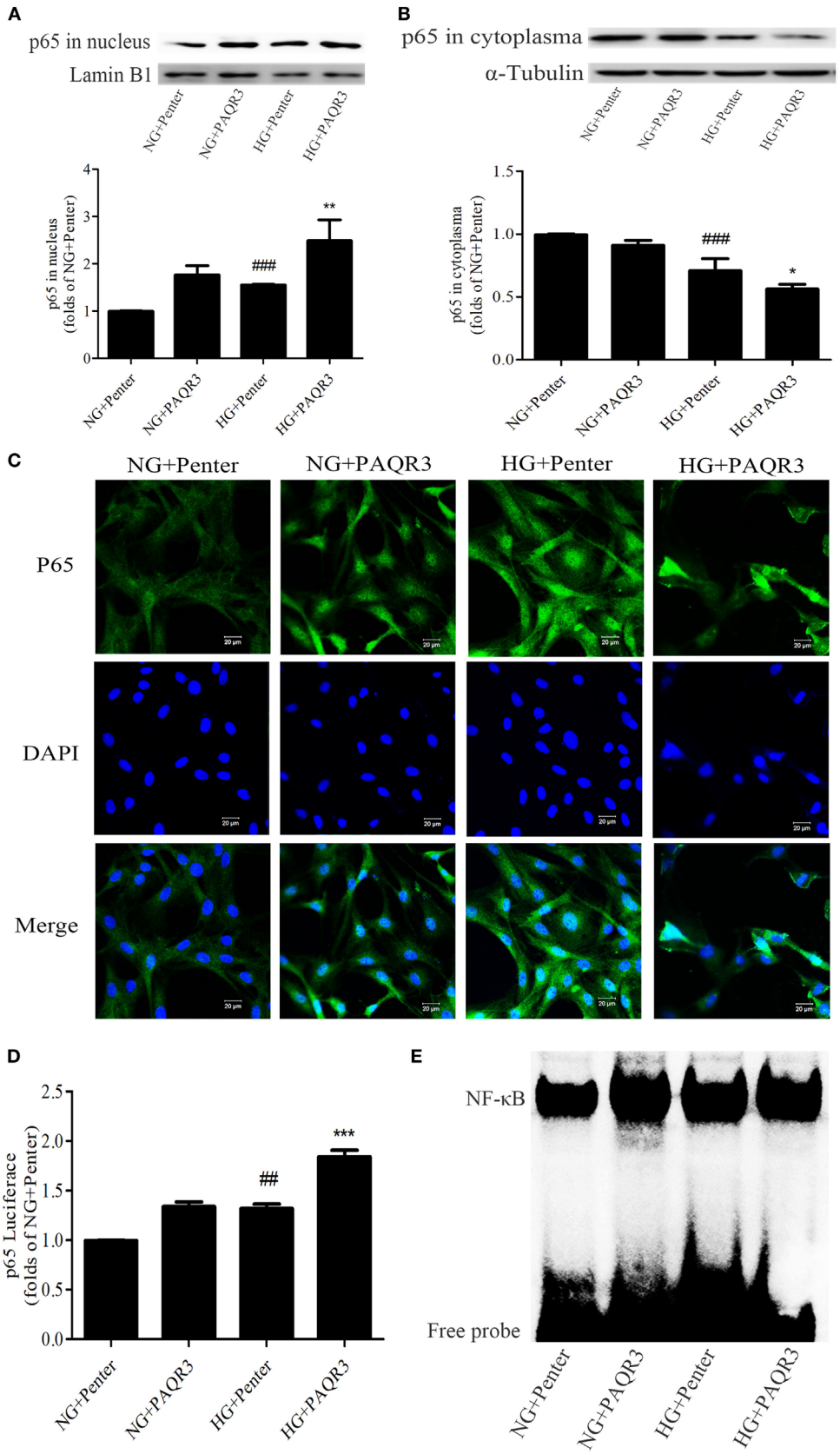

FIGURE 5 | Continued

FIGURE 5 | Upregulation of progestin and adipoQ receptor 3 (PAQR3) further increased the NF-κB nuclear translocation, DNA binding activity and transcriptional activity in glomerular mesangial cells. Overexpression of PAQR3 increased nuclear level of p65 **(A)** and decreased its cytoplasm level **(B)**. $^{###}P < 0.001$ vs. NG + Penter, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. high glucose (HG) + Penter. PAQR3 overexpression enhanced the nuclear accumulation of p65, which was measured by immunofluorescent staining **(C)**. Bar: 20 μm. The effect of PAQR3 overexpression further enhanced the transcriptional activity of p65, which was obtained by luciferase reporter assay **(D)**. $^{##}P < 0.01$ vs. NG + Penter, $^{***}P < 0.001$ vs. HG + Penter. Electrophoretic mobility shift assay was performed to determine the elevated DNA binding activity of p65 **(E)**. Independent experiments were performed at least three times with similar results.

This increased nuclear translocation was further confirmed in immunofluorescence images (**Figure 5C**). Dual luciferase reporter assay and EMSA also showed that transcriptional activity and DNA binding activity of NF-κB were further enhanced after overexpression of PAQR3 (**Figures 5D,E**). Taken together, our data indicated that overexpression of PAQR3 further activates NF-κB pathway in GMCs under HG conditions.

PAQR3 Inhibition Suppressed the NF-κB Nuclear Translocation, DNA Binding, and Transcriptional Activity in GMCs

To further confirm the effect of PAQR3 on NF-κB pathway, the nuclear levels, DNA binding, and transcriptional activity of NF-κB were measured after silencing PAQR3. As expected, knockdown of PAQR3 suppressed HG-induced NF-κB nuclear translocation in GMCs (**Figures 6A–C**). Subsequently, the transcriptional activity (**Figure 6D**) and DNA binding activity (**Figure 6E**) of NF-κB were attenuated after PAQR3 knockdown, as confirmed by dual luciferase reporter assay and EMSA results. In general, our data indicated that PAQR3 positively regulates NF-κB signaling pathway in GMCs under HG conditions.

HG Stimulation Attenuated the Interaction Between PAQR3 and IKKβ

Progestin and adipoQ receptor 3 and NF-κB inflammatory signaling pathway all play important roles in the development of DN. To further study the relationship between PAQR3 and NF-κB, co-immunoprecipitation (Co-IP) assay was performed to investigate the mechanism underlying the regulation of PAQR3 on NF-κB signaling pathway. IKKβ is an upstream regulator of NF-κB signaling pathway, which can activate NF-κB pathway through phosphorylation of IκB (inhibitor of NF-κB) protein. By Co-IP assay, we found that PAQR3 interacted with IKKβ under physiological status. Moreover, this interaction was diminished after HG stimulation (**Figure 7**). Therefore, our data suggested that HG stimulation attenuates the interaction between PAQR3 and IKKβ, resulting in translocation of IKKβ to cytoplasm to phosphorylate IκB.

Genetical and Pharmacological Inhibition of NF-κB Attenuated the Effects of PAQR3 Overexpression on HG-Induced Upregulation of FN and ICAM-1

To further confirm whether NF-κB pathway was involved in the effect of PAQR3 on regulation of FN and ICAM-1, we both genetically knocked down NF-κB by using siRNA(**Figures 8A,B**), and pharmacologically inhibit NF-κB by using PDTC(**Figures 8C,D**), a well characterized inhibitor of NF-κB (31, 32). Overexpression of PAQR3 could further enhance the HG-induced upregulation of FN and ICAM-1 in GMCs. Transfection of si-NF-κB or treatment with PDTC alone significantly abrogated these effects. While GMCs were transfected with si-NF-κB or treated with PDTC in the presence of PAQR3 overexpression, the expressions of FN and ICAM-1 had no obvious changes compared with genetical and pharmacological inhibition of NF-κB alone. Therefore, our data suggested that PAQR3 mediates expressions of FN and ICAM-1 in DN *via* activation of NF-κB pathway.

DISCUSSION

PAQR family consists of 11 human PAQRs membrane proteins receptors, which can be grouped into three main classes, adiponectin receptors related subgroup (PAQR1-PAQR4), progesterone membrane protein receptors related subgroup (PAQR5-PAQR9), and the rest (PAQR10-PAQR11), based on the sequence comparisons (23, 33). PAQR3 is a type III topology receptor protein with a cytosolic N-terminus. Both N-terminus and loop structure are critical for PAQR3 to interact with signaling molecules in cytoplasm, thus to regulate the transmission of intracellular signals (22, 34). Since PAQR3 can be activated by adiponectin and shares high sequence homology with PAQR1 and PAQR2, it is classified into the adiponectin receptors related subgroup. PAQR3, as a new tumor suppressor gene, plays a vital role in inflammation, insulin resistance, glucose and lipid metabolic disorder diseases (23, 24, 35–39). It is also found that PAQR3 knockout obviously reversed HFD-induced diabetes, fatty liver, and remarkably improved insulin resistance, strengthen energy metabolism in mice (25). All of these evidences suggested PAQR3 plays an important role in the progression of diabetes and other diabetes-related complications.

Our study demonstrated that PAQR3 protein level was significantly increased by 4.75- and 4.5-fold in the kidneys of STZ-induced diabetic mice and rats, respectively. Also, HG stimulation increased the level of PAQR3 in GMCs in a time-dependent manner. While overexpression of PAQR3 further increased the HG-induced upregulation of FN and ICAM-1, PAQR3 silencing reversed these upregulation in GMCs. These results suggested that PAQR3 plays a role in the development of renal fibrosis in DN.

NF-κB signaling pathway is one of the classic inflammatory response pathways with the main function of regulating a large inflammatory gene in DN (20, 40). As we proved that PAQR3-silencing ameliorated the expressions of FN and ICAM-1, we wondered whether NF-κB pathway was involved in the regulation of PAQR3. As confirmed by multiple approaches,

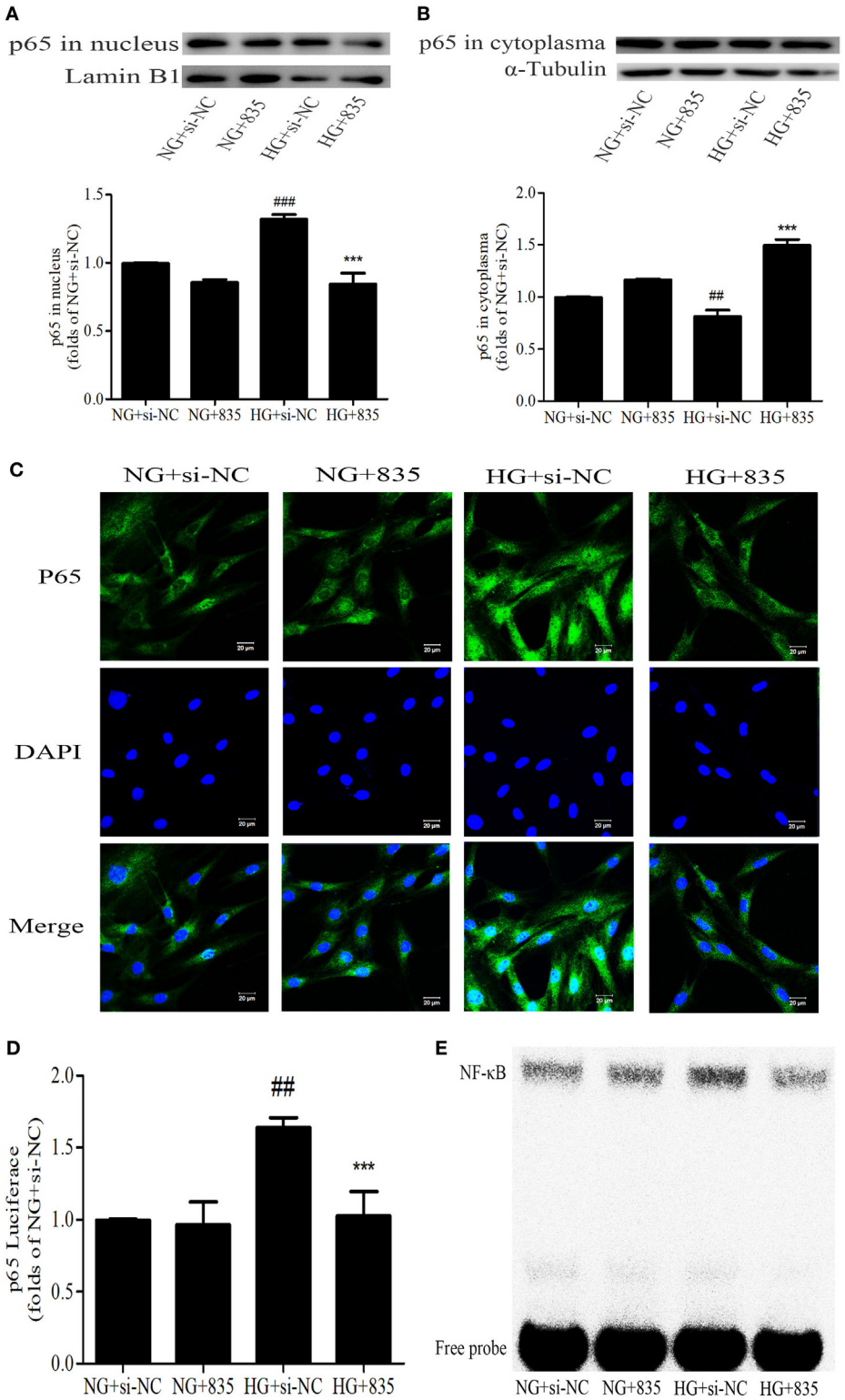

FIGURE 6 | Progestin and adipoQ receptor 3 (PAQR3) inhibition suppressed the NF-κB nuclear translocation, DNA binding, and transcriptional activity in glomerular mesangial cells. High glucose (HG) stimulation increased p65 nuclear translocation, treatment with si835 decreased the nuclear level of p65 **(A)** and increased its cytoplasm level **(B)**. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. NG + si-NC, $^{***}P < 0.001$ vs. HG + si-NC. HG-induced nuclear accumulation of p65 was prohibited under the conditions of PAQR3 inhibition **(C)**. Bar: 20 μm. HG stimulation for 12 h increased p65 transcriptional activity, which was inhibited by treatment with si835 **(D)**. $^{\#\#}P < 0.01$ vs. NG + si-NC, $^{***}P < 0.001$ vs. HG + si-NC. Knockdown of PAQR3 decreased the DNA binding activity of p65 compared with HG group **(E)**. Independent experiments were performed at least three times with similar results.

overexpression of PAQR3 increased nuclear accumulation, transcriptional activity, and DNA binding activity of NF-κB in HG-treated GMCs. In contrast, silencing of PAQR3 suppressed HG-induced augment of nuclear accumulation, transcriptional activity, and DNA binding activity of NF-κB in GMCs. Moreover, knockdown of NF-κB or treatment with NF-κB inhibitor, PDTC, abrogated the effect of PAQR3 overexpression on upregulation of FN and ICAM-1. In sum, our results indicated that PAQR3 regulates expressions of FN and ICAM-1 *via* NF-κB pathway.

However, the accurate mechanism how PAQR3 regulates activation of NF-κB pathway in DN remains to be elucidated. It was reported that IKKβ/NF-κB signaling pathway plays a major role in the metabolic inflammation (41, 42). IKKβ/NF-κB, as a mediator of metabolic inflammation, is a new strategy to combat obesity and its related diseases *via* regulating central insulin/leptin signaling and action (43–45). In light of reports that the IKKβ is required for the activation of NF-κB (46–48), we explored the interaction between PAQR3 and IKKβ in physiological status, or under the conditions of HG stimulation using co-IP. Here, we first reported the direct interaction between PAQR3 and IKKβ in physiological conditions, while the interaction was attenuated under conditions of HG stimulation. As it is well known that IKKβ is an upstream mediator of NF-κB signaling pathway, PAQR3 regulated NF-κB might *via* its interaction with IKKβ. However, the mechanism remained to be further explored. Considering that PAQR3 is also known as a Golgi-anchored membrane protein and Golgi apparatus is a workshop for proteins, we suspected that PAQR3 and IKKβ may co-exist in Golgi membrane as inactive forms. While stimulated by HG, IKKβ was separated from PAQR3 and moved to cytoplasm. Free and active IKKβ phosphorylated IκB to increase its degradation, resulting in translocation of NF-κB into nucleus to regulate the expression of downstream inflammatory genes.

In summary, our present study demonstrated that PAQR3 mediated pathogenesis of diabetic renal inflammatory fibrosis through NF-κB signaling pathway. Further investigation will be needed to determine if PAQR3 is a potential target for the treatment of diabetic kidney fibrosis.

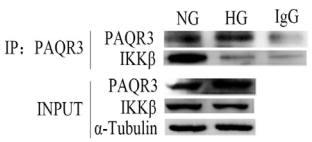

FIGURE 7 | High glucose (HG) stimulation attenuated the interaction between progestin and adipoQ receptor 3 (PAQR3) and IKKβ. Immunoprecipitation assay was applied to detect the interaction between PAQR3 and IKKβ under NG and 1 h of HG treatment. Independent experiments were performed at least three times with similar results.

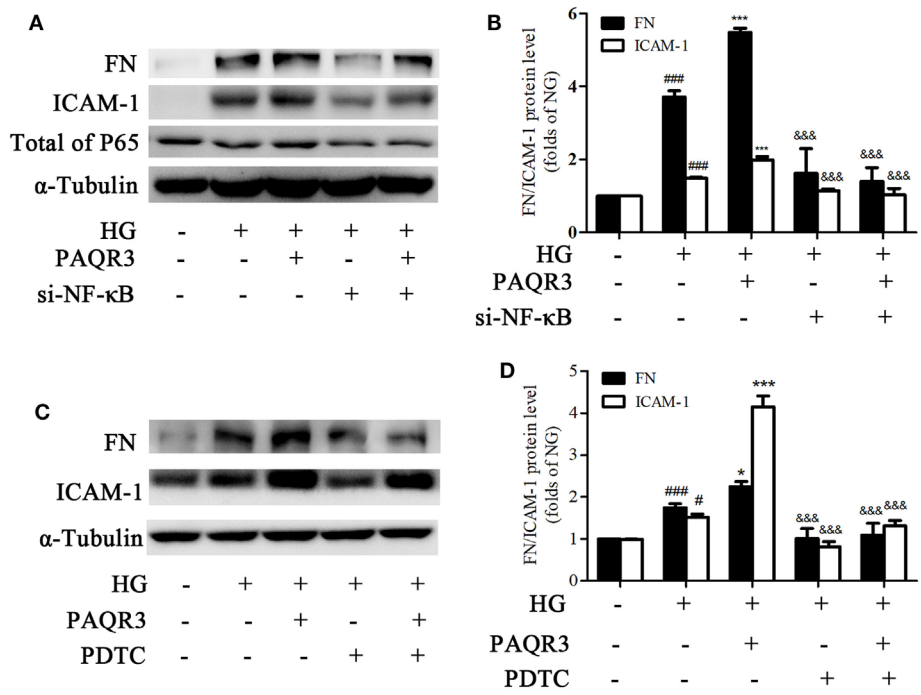

FIGURE 8 | Genetical and pharmacological inhibition of NF-κB attenuated the effects of progestin and adipoQ receptor 3 (PAQR3) overexpression on high glucose (HG)-induced upregulation of fibronectin and intercellular adhesion molecule-1. Glomerular mesangial cells (GMCs) were transfected with si-NF-κB and PAQR3 plasmid for 24 h, then treated with HG for 24 h and total proteins were harvested to Western blot assay **(A,B)**. $^{###}P < 0.001$ vs. NG, $^{***}P < 0.001$ vs. HG, $^{\&\&\&}P < 0.001$ vs. HG + PAQR3. GMCs were transfected with PAQR3 plasmid for 24 h, and then treated with PDTC (10 μM) under HG conditions for 24 h **(C,D)**. Total proteins were harvested to Western blot assay. $^{\#}P < 0.05$, $^{###}P < 0.001$ vs. NG, $^{*}P < 0.05$, $^{***}P < 0.001$ vs. HG, $^{\&\&\&}P < 0.001$ vs. HG + PAQR3. Independent experiments were performed at least three times with similar results.

ETHICS STATEMENT

All experimental procedures were carried out in accordance with the China Animal Welfare Legislation, and approved by the Ethics Committee on the Care and Use of Laboratory Animals of Sun Yat-sen University.

AUTHOR CONTRIBUTIONS

YZ was mainly responsible for the idea of the article, conducting experiments, analyzing data, and writing articles. ZC participated in the language polish and article revision. JL participated in the data analysis and article revision. WG was mainly joined in experimental design and LZ involved in processing data mostly, and less other works. FX and LC major joined in experimental execution, and less other works. PL is our supervisor and involved in the idea of the article. HH is our supervisor. Besides, he was also involved in all parts of our research, such as providing ideas, instructing experiments and articles.

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

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Circulating Endothelial Progenitor Cells in Type 1 Diabetic Patients: Relation with Patients' Age and Disease Duration

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Objectives: Circulating endothelial progenitor cells (cEPCs) have been reported to be dysfunctional in diabetes mellitus (DM) patients, accounting for the vascular damage and the ensuing high risk for cardiovascular disease (CVD) characteristic of this disease. The aim of the present study was to evaluate the number of circulating cEPCs in type 1 DM (T1DM) patients, without clinical vascular damage, of different ages and with different disease duration.

Methods: An observational, clinical-based prospective study was performed on T1DM patients enrolled in two clinical centers. cEPCs were determined by flow cytometry, determining the number of CD34/CD133/VEGFR2-positive cells within peripheral blood mononuclear cells (PBMCs).

Results: The number of cEPCs was lower in adult T1DM patients, whilst higher in childhood/young patients, compared to controls of the same age range. When patients were grouped into two age groups (≥ or <20 years) (and categorized on the basis of the duration of the disease), the number of cEPCs in young (<20 years) patients was higher compared with older subjects, regardless of disease duration. A subset of patients with very high cEPCs was identified in the <20 years group.

Conclusion: There is an association between the number of cEPCs and patients' age: childhood/young T1DM patients have significantly higher levels of cEPCs, respect to adult T1DM patients. Such difference is maintained also when the disease lasts for more than 10 years. The very high levels of cEPCs, identified in a subset of childhood/young patients, might protect vessels against endothelial dysfunction and damage. Such protection would be less operative in older subjects, endowed with lower cEPC numbers, in which complications are known to develop more easily.

Keywords: type 1 diabetes mellitus, endothelial progenitor cells, flow cytometry, diabetes duration, patients' age

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INTRODUCTION

Diabetes mellitus (DM) is characterized by long-term vascular damage to small vessels and major arteries and by an impaired vascular repair, which collectively leads to a higher risk of cardiovascular disease (CVD) (1). Contributory factors to the vascular impairment in DM include increased glucose level, other traditional cardiovascular risk factors, arterial wall inflammation,

and endothelial dysfunction (2). Endothelial dysfunction is considered the pivotal mechanism sustaining vascular injury, and hence the heightened cardiovascular burden in DM (3).

After a vascular injury, endothelial repair depends both on the migration and proliferation of endothelial cells of the vascular wall and by the arrival of endothelial progenitor cells (EPCs) from the bone marrow in the site of damage (4, 5). The release of EPCs from the bone marrow depends on the stimulatory effect of different growth factors/cytokines, such as VEGF and IL-8 (6, 7). In this light, a novel paradigm of CVD pathogenesis is the loss of normal endothelial turnover caused by a reduction of EPCs [reviewed by Shantsila et al. (8)].

A reduction of circulating EPCs (cEPCs) has been hypothesized to promote the development and/or progression of vascular dysfunction and CVD in DM (9). EPC dysfunction would also represent the molecular transducer in the mechanism through which risk factors negatively affect cardiovascular function in DM (10). Consistently, several reports have shown that both the number and functionality of cEPCs are reduced in type 2 DM (T2DM) and that such impairment is related to the morphological and functional alterations detected in peripheral vessels (9, 11–13). Furthermore, T2DM patients show low serum levels of those growth factors/cytokines known to trigger EPC release from the bone marrow, accounting for a reduced bone marrow stimulation and hence EPCs release (14). The levels of cEPCs and arterial wall stiffness in T2DM subjects are strictly correlated with glycemic control (15, 16). Interestingly, a clear correlation between EPC activity, glycemic control, and myocardial savage has been recently demonstrated (17). Hyperglycemia may *per se* affect EPC number and functional capacity, because it enhances EPC senescence and triggers apoptosis (18). Sirtuins have been identified as molecular mediators of the deleterious effect of hyperglycemia in EPCs (16, 19).

The scenario is apparently similar in Type 1 DM (T1DM), where a general reduction of cEPC number has been reported (20–24). Only Głowińska-Olszewska et al. (25) showed that contrary to adult population with diabetes, T1DM diabetic children have an increased number of EPCs.

Based on the latter data and on the clinical observation of less incidence of late vascular complications in T1DM when the onset of the disease is in childhood respect to adult age (26), we undertook a study aimed at determining the number of cEPCs in T1DM patients without clinical vascular damage, of different ages and disease duration.

MATERIALS AND METHODS

Study Population

We performed an observational, clinical-based prospective study on type 1 diabetic patients treated at two different Italian centers: the Diabetic Unit of the Meyer Hospital in Florence and the Diabetic Unit of the Prato Hospital, Prato. The Meyer Hospital specifically enrolled childhood (age <10 years) and young (age 10–24 years) T1DM patients and age-matched controls; the Diabetic Unit of the Prato Hospital enrolled adult (25–59 years) T1DM patients and age-matched controls. Patients were enrolled after informed written consent in accordance with the Declaration of Helsinki. The study was approved by Local Ethical Committee. The enrollment started in January 2010 and ended on May 2012; patients were followed up until December 2014. Inclusion criteria were the clinical diagnosis of T1DM from at least two years and the lack of clinical CV complications. In particular, patients were screened for hypertension, coronary artery disease, peripheral arterial disease, peripheral neuropathy, retinopathy, and nephropathy.

Twenty-two healthy individuals, selected within the same age range as T1DM patients, were enrolled as controls. None of the controls had a clinical history of diabetes. They had normal fasting blood glucose levels and normal physical examination and had not received any medication.

Sample Preparation

Mononuclear cells from peripheral blood mononuclear cell (PBMC) samples were isolated by density gradient centrifugation using Lympholyte (Cedarlane Laboratories, Burlington, ON, Canada). Briefly, 5 ml of peripheral blood were diluted 1:2 with PBS and the diluted blood was stratified onto 5 ml of Lympholyte. Samples were centrifuged 30 min at room temperature at 3,000 rpm without brake. After separation, white blood cells were recollected, diluted with PBS and centrifuged at 1,200 rpm for 5 min at room temperature. Subsequently, pellet was treated with Red Cell Lysis Buffer, to ensure red blood cell removal and washed in PBS.

Fluorescence-Activated Cell Sorting (FACS) Analysis

The number of cEPCs was assessed by flow cytometry by determining the number of CD34/CD133/VEGFR2-positive cells. In particular, 100 μl of each sample prepared as described in the previous section, were stained with 1 μl each of FITC-CD34 (BD Biosciences, Franklin Lakes, NJ, USA), APC-CD133/2 (Miltenyi Biotec, Bergisch Gladbach, Germany), and PE-VEGFR-2 (R&D Systems, Minneapolis, MN, USA), and incubated in the dark in ice for 15 min. For each sample, a control tube with no antibodies was prepared together with the stained tube. After washing the cells with PBS, FACS analysis was performed on a FacsCanto (BD Biosciences, Franklin Lakes, NJ, USA). The acquisition goal was 2×10^5 events. Samples were analyzed gating a population with morphological characteristics between lymphocytes and monocytes, evaluated on the basis of side scatter and forward scatter parameters. As evident from **Figure 1**, where representative dot-plots relative to a control and a T1DM patient (belonging to the "<20 y T1DM cohort") are shown, the cEPC count is reported in Q2, that is a part of the P4 quadrant. Throughout the manuscript, "cEPC counts" refers to the absolute cEPC number per 2×10^5 PBMC.

Statistical Analysis

Data are given as mean ± SEM. First of all, normality of the distribution of cEPC counts was assessed by Kolmogorov–Smirnov test. In order to apply the correct type of *T* test (for

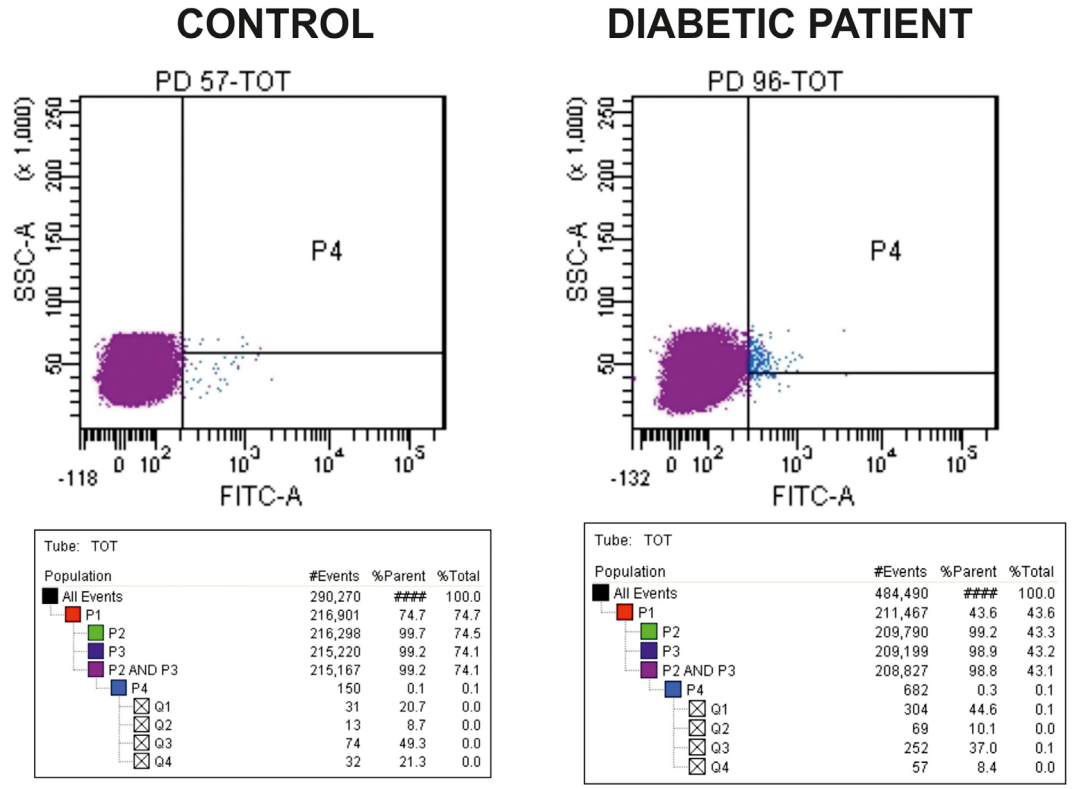

FIGURE 1 | Representative dot-plots of a control subject [left panel, circulating endothelial progenitor cells (cEPCs) = 8.7] and diabetic patient belonging to the "<20 y T1DM" cohort (right panel, cEPCs = 10.1). The cEPC count was performed as described in the Section "Materials and Methods" and gating a population with morphological characteristics between lymphocytes and monocytes (evaluated through side scatter and forward scatter). In the final plots, the cEPC count of each sample is reported in Q2 that is a part of P4 quadrant. "cEPC counts" refers to the absolute cEPC number per 2×10^5.

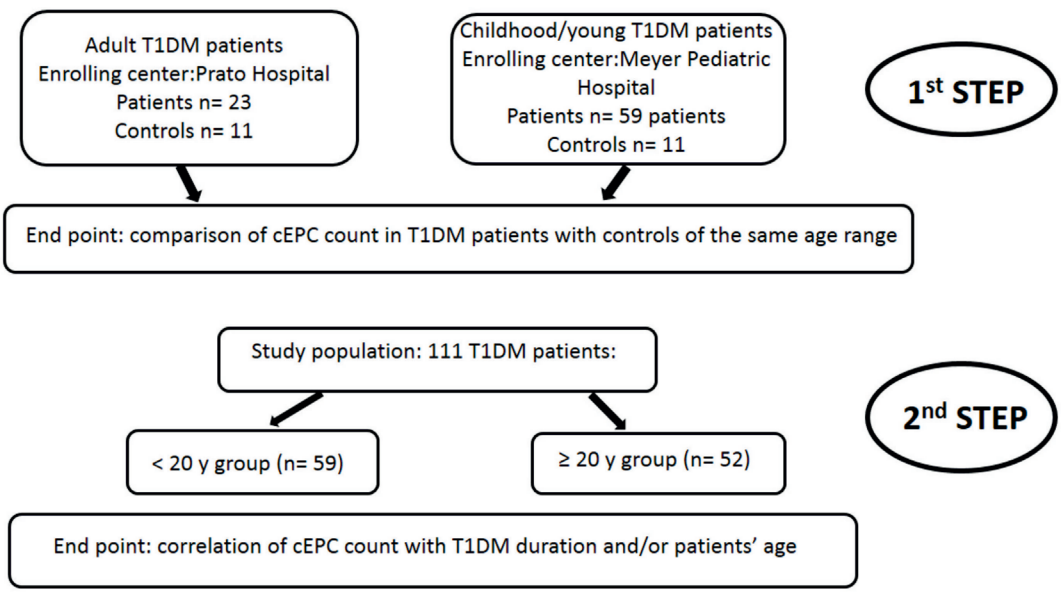

FIGURE 2 | Flow diagram showing the rationale of the study. The end point of the first step of the study was the evaluation of the number of circulating endothelial progenitor cells (cEPCs) in adult and childhood/young patients, compared to age-matched controls. In the second step of the study, the end point was the association of the number of cEPCs with disease duration and/or patients' age.

unpaired samples with equal or different variance) for normally distributed samples, the analysis of the variance was assessed by ANOVA test at the 0.05 level. Once determined the normality as well as the variance of the samples, the proper test was applied to evaluate differences among groups. In particular, we used the two-sided Student's *t*-test for unpaired samples (either with different variance or with equal variance) for normally distributed data and the Mann–Whitney *U* test when samples were not normally distributed. In both cases, $p < 0.05$ was considered as significant. The Pearson correlation coefficient was calculated to evaluate relationships between cEPC count and clinical parameters.

Study Design

As depicted in the flow diagram shown in **Figure 2**, the study was divided into two steps. In the first step, two cohorts of T1DM patients enrolled in the two (adult and pediatric) diabetic centers were analyzed independently. The end point of the first step of the study was the evaluation of the number of cEPCs in adult and childhood/young patients, compared to controls of the same age range. In the second step, the study population (a total of 111 patients enrolled in both centers) was divided in two groups of similar numerosity, depending on the age (≥ or <20 years). The end point of this second step was the analysis of the association between the number of cEPCs and disease duration and/or patients' age.

RESULTS

The first objective of our study was to determine the number of cEPCs in T1DM patients compared to healthy controls (see the flow diagram of the study in **Figure 2**). Two different patients' cohorts were examined: one relative to adult T1DM patients enrolled in the Prato Hospital and one relative to childhood/young patients, enrolled in the Meyer Pediatric Florence Hospital. The clinical characteristics of T1DM patients enrolled in the two centers are shown in **Tables 1** and **2**, along with cEPC data. Note that for healthy controls, only gender and age data are reported in the tables. The number of cEPCs was significantly reduced in adult T1DM patients compared

TABLE 1 | Clinical characteristics of adult type 1 diabetes mellitus (T1DM) patients and control subjects enrolled for the setting up of circulating endothelial progenitor cell (cEPC) detection and evaluation.

Patient ID	Gender	T1DM duration (years)	cEPC	Age	HbA1c (%)	BMI	Body weight (kg)	Height (cm)	Fat mass %	Lean mass %	CHO	HBGI	LBGI
A01	Female	13	6.1	45	7.0	19.03	55.0	170	22.2	77.8	Yes	11.5	1.5
A02	Female	9	5.4	38	8.7	28.08	91.0	180	20.8	79.2	Yes	10.8	1.4
A03	Female	4	6.3	38	8.1	23.14	63.0	165	27.7	72.3	Yes	10.6	1.3
A04	Male	20	4.0	47	7.2	27.40	96.0	190	24.0	76.0	Yes	3.8	2.8
A05	Female	33	8.4	33	7.2	18.80	53.0	168	18.1	81.9	Yes	11.1	1.6
A06	Male	26	8.1	43	7.2	21.90	67.0	175	19.2	80.8	Yes	7.2	4.1
A07	Female	26	3.2	59	7.3	20.60	61.0	172	26.4	73.6	Yes	4.15	5.17
A09	Female	5	4.2	37	8.4	22.60	53.0	153	27.3	72.7	Yes	11.3	1.6
A10	Female	6	3.3	31	7.4	21.10	55.4	162	26.4	73.6	Yes	8.6	6.5
A11	Female	23	2.8	35	7.4	27.10	65.0	155	26.7	73.3	Yes	6.2	3.8
A12	Female	21	3.9	36	6.8	20.40	61.0	173	26.3	73.7	Yes	4.3	4.2
B01	Male	2	5.8	22	7.0	22.78	72.0	178	11.4	88.6	No	10.1	6.5
B02	Male	32	1.2	59	7.5	20.22	53.0	162	17.4	82.6	No	12.2	1.9
B03	Female	28	0.6	40	8.5	25.17	70.0	167	30.5	69.5	No	10.8	1.4
B04	Female	2	4.4	28	6.8	21.09	52.0	157	19.2	80.8	No	11.3	1.6
B05	Male	2	5.6	27	6.5	24.44	74.0	174	26.4	73.6	No	10.8	1.5
B06	Female	2	4.8	25	7.0	18.53	48.0	161	11.5	88.5	No	3.8	2.8
B07	Male	10	4.4	24	7.5	27.04	85.0	188	23.1	76.9	No	12.1	1.8
B08	Female	22	5.1	37	7.0	21.30	61.0	170	19.4	80.6	No	6.2	3.0
B09	Female	2	5.3	43	10	20.70	55.0	163	20.1	79.9	No	10.6	1.3
B10	Male	11	6.0	39	6.5	25.4	75.0	172	26.3	73.7	No	10.3	1.2
B11	Female	6	0.8	42	8.0	22.3	60.0	164	23.3	76.7	No	11.6	1.5
B12	Female	22	2.2	39	6.4	22.00	65.0	172	26.4	73.6	No	7.6	4.5
C01	Male	Control	11.2	48									
C02	Female	Control	9.4	39									
C03	Male	Control	8.2	51									
C04	Male	Control	7.9	46									
C05	Male	Control	9.4	45									
C06	Female	Control	11.0	28									
C07	Male	Control	11.4	38									
C08	Male	Control	11.8	46									
C09	Male	Control	11.6	48									
C10	Female	Control	10.9	49									
C11	male	Control	10.4	37									

BMI, body mass index; CHO, carbohydrate counting; HBGI, high blood glucose index; LGBI, low blood glucose index.

TABLE 2 | Clinical characteristics of childhood/young type 1 diabetes mellitus (T1DM) patients enrolled in the study.

Patient ID	Gender	T1DM duration (years)	cEPC	Age	HbA1c	BMI	Glycemia	I/W
PD 001	Male	14	9.2	19	6.5	21.10	67	0.79
PD 002	Male	4	32.6	17	6.6	ND	100	0.68
PD 003	Male	4	16.7	8	6.8	16.40	164	0.65
PD 004	Female	6	9.2	18	8.5	21.60	239	1.16
PD 005	Male	5	2.3	13	9.0	17.30	205	0.74
PD 010	Female	4	8.3	15	7.1	25.70	197	0.45
PD 012	Male	2	0.7	8	7.3	16.40	182	0.84
PD 013	Female	12	11.7	16	8.3	22.90	226	0.82
PD 020	Male	3	0.3	7	9.7	ND	250	ND
PD 021	Male	9	3.0	19	9.8	24.40	90	0.75
PD 022	Male	I4	43.3	19	8.6	24.40	271	0.62
PD 025	Female	10	3.6	14	7.8	21.40	142	1.00
PD 032	Male	5	4.5	7	7.5	14.50	119	0.70
PD 033	Female	1	1.8	15	6.3	25.10	126	0.72
PD 035	Male	2	40.3	17	5.7	23.60	186	0.40
PD 037	Female	4	6.8	14	8.2	17.70	212	0.90
PD 040	Male	2	2.7	12	7.6	20.70	107	0.93
PD 041	Male	2	11.9	8	7.5	15.80	114	0.67
PD 042	Female	14	23.4	17	7.5	18.00	289	1.03
PD 044	Female	2	75.0	16	6.5	24.80	108	0.88
PD 045	Male	2	10.2	9	6.5	16.90	122	0.76
PD 046	Male	5	18.3	18	6.7	19.40	179	0.83
PD 047	Male	8	4.5	18	7.2	23.20	164	0.70
PD 048	Female	2	7.6	16	6.0	20.90	117	1.14
PD 049	Female	1	14.0	9	8.0	23.40	208	1.28
PD 050	Female	5	10.6	14	8.0	29.90	102	0.92
PD 051	Female	9	16.4	13	7.4	18.00	304	1.05
PD 054	Male	10	45.8	15	9.5	24.10	220	1.08
PD 055	Female	4	17.5	17	7.7	ND	365	ND
PD 056	Female	5	17.9	10	8.1	15.60	368	0.74
PD 058	Male	4	20.2	15	10.3	ND	270	0.82
PD 063	Female	4	38.3	17	7.5	26.90	164	0.84
PD 064	Male	11	8.4	14	8.1	17.80	83	0.71
PD 065	Female	9	19.0	10	7.5	14.40	174	0.92
PD 066	Male	4	24.2	11	8.0	18.10	205	0.92
PD 067	Male	8	38.6	11	7.3	17.40	102	1.02
PD 068	Female	2	30.4	10	8.7	19.70	203	1.08
PD 069	Female	4	11.3	14	7.2	21.60	270	0.98
PD 070	Female	12	34.4	19	8.7	27.10	163	0.61
PD 071	Male	1	26.8	10	6.8	17.20	146	0.89
PD 073	Male	11	2.4	18	7.3	22.80	183	0.73
PD 074	Male	12	0.3	17	7.0	29.30	106	0.86
PD 075	Male	4	3.3	18	6.1	ND	155	ND
PD 080	Male	5	1.4	16	10.2	19.90	333	1.16
PD 081	Male	8	16.5	14	7.0	30.40	319	0.90
PD 083	Female	9	8.2	13	7.8	18.20	334	1.00
PD 084	Female	2	12.5	17	6.4	22.30	104	0.69
PD 085	Female	1	25.7	14	8.0	23.30	181	0.54
PD 086	Male	6	35.0	10	8.0	15.60	165	0.55
PD 087	Female	1	2.9	6	6.8	15.40	223	0.07
PD 088	Male	10	7.1	13	8.0	17.20	210	1.06
PD 090	Male	8	23.4	15	7.8	19.20	178	0.93
PD 091	Male	1	2.6	16	8.0	21.60	243	0.40
PD 093	Female	2	3.7	10	7.6	17.50	173	0.82
PD 094	Male	4	6.2	16	9.0	23.80	279	0.77
PD 097	Female	2	3.5	16	7.7	26.10	185	0.58
PD 099	Male	13	42.0	19	7.9	ND	54	ND
PD 101	Female	10	4.2	14	7.0	21.50	71	1.06
PD 112	Male	2	5.3	10	7.2	19.10	243	0.74
PD 006	Male	Control	18	14				
PD 007	Female	Control	3.1	15				
PD 008	Male	Control	4.0	11				
PD 017	Female	Control	2.3	15				
PD 018	Male	Control	1.5	7				

(*Continued*)

TABLE 2 | Continued

Patient ID	Gender	T1DM duration (years)	cEPC	Age	HbA1c	BMI	Glycemia	I/W
PD 019	Male	Control	3.6	8				
PD 057	Male	Control	8.7	3				
PD 103	Male	Control	6.2	7				
PD 108	Female	Control	0.2	11				
PD 113	Male	Control	1.7	18				
PD 114	Male	Control	4.8	7				

BMI, body mass index; I/W, insulin/weight.

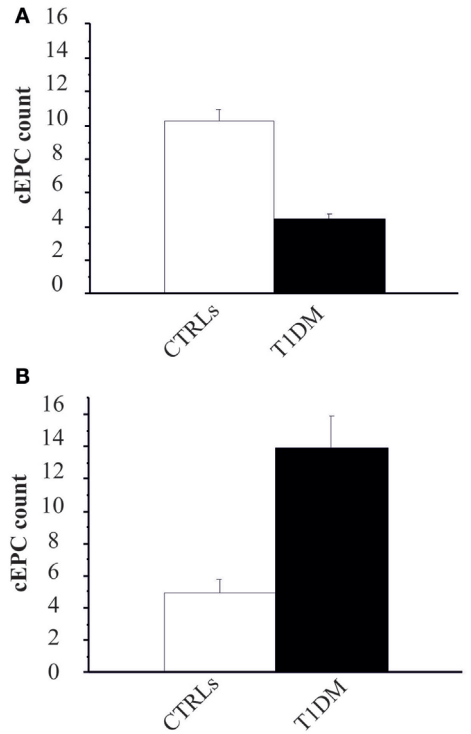

FIGURE 3 | **(A)** Circulating endothelial progenitor cell (cEPC) counts (absolute cEPC number per 2×10^5 peripheral blood mononuclear cell) in adult control subjects (white bar) and adult type 1 diabetes mellitus (T1DM) (black bar). Data are reported as mean ± SEM; $p < 0.001$, Student's *t*-test. **(B)** cEPC count in childhood/young control subjects (white bar) and age-matched T1DM (black bar). Data are reported as mean ± SEM; $p < 0.001$, Mann–Whitney *U* test.

TABLE 3 | Clinical characteristics of adult and childhood/young type 1 diabetes mellitus (T1DM) patients enrolled to complete the cohort under study.

Center	Patient ID	T1DM duration (years)	cEPC	Age
PO	AD 001	14	7.5	49
PO	AD 002	12	5.6	50
PO	AD 003	14	9.7	40
PO	AD 004	12	3.1	47
PO	AD 005	18	11.2	35
PO	AD 006	11	4.3	43
PO	AD 007	10	1.4	32
PO	AD 008	13	3.4	38
PO	AD 009	20	3.6	38
PO	AD 010	18	4.8	32
PO	AD 011	22	9.3	56
PO	AD 012	28	5.8	39
PO	AD 013	30	8.4	42
PO	AD 015	32	6.6	42
AOUM	PD 011	5	1.2	24
AOUM	PD 023	18	4.1	21
AOUM	PD 029	6	66.2	22
AOUM	PD 031	12	12.5	20
AOUM	PD 036	14	10.4	27
AOUM	PD 038	13	22.3	23
AOUM	PD 052	12	11.1	21
AOUM	PD 059	12	15.0	20
AOUM	PD 060	12	13.1	22
AOUM	PD 092	12	1.2	25
AOUM	PD 096	9	10.1	22
AOUM	PD 100	11	8.0	21
AOUM	PD 109	39	1.5	42
AOUM	PD 111	11	0.6	20

to controls within the same age range (4.43 ± 0.29 $n = 23$ vs 10.29 ± 0.68 $n = 11$; $p < 0.001$, Student's *t*-test) (**Figure 3A**, raw data are in **Table 1**). On the contrary, childhood/young T1DM patients had significantly higher levels of cEPCs (13.90 ± 1.95 $n = 59$) compared to control subjects (4.92 ± 0.89 $n = 11$; $p < 0.001$, Mann–Whitney *U* test) (**Figure 3B**, raw data are in **Table 2**). In both cohorts, no statistically significant association emerged between the number of cEPCs and available clinical parameters, such as gender, percentage of glycated Hb, and body mass index (BMI).

We then enrolled 28 T1DM patients from either centers (**Table 3**), so that the study population was: 52 patients in the ≥20 years group and 59 in the <20 years group. In order to evaluate whether the number of cEPCs was anyhow related to the patients' age and/or with the duration of the disease T1DM patients from both centers were grouped into two age categories: patients younger (<20 years) or older than 20 years (≥20 years). In agreement with data shown in **Figure 3**, the number of cEPCs turned out to be significantly higher in T1DM patients younger than 20 years with respect to older patients (15.24 ± 1.12 $n = 59$ vs 7.27 ± 0.73 $n = 52$; $p = 0.004$, Mann–Whitney *U* test) (**Figure 4A**).

Once demonstrated that the number of circulating EPCs falls with the increasing of age, we evaluated whether the differences in cEPCs levels depended only on the age of the patient or also on the duration of the disease. To this purpose, we categorized T1DM patients into the two groups depending either on the age (≥ or <20 years) or on the duration of the disease (shorter or longer than 10 years, DD < 10 years or DD ≥ 10 years) and divided each group into two subgroups, based on DD and age,

respectively. No differences were found between DD subgroups when the patients were categorized by age (**Figure 4B**, left panel; individual means and *p* values are in figure legend). When the patients were categorized on the basis of disease duration, the difference in cEPC counts between <20 and ≥20 years patients was roughly of the same entity in both DD groups, although with a lower *p* value in the DD ≥ 10 years group (**Figure 4B**, right panel; individual means and *p* values are in figure legend). Furthermore, we plotted the individual cEPC count values vs either patients' age or duration (**Figure 5**). A subset of childhood/young patients, aged less than 20 years (and with a disease duration less than 10 years) emerged with very high cEPC counts (black shaded circles in **Figure 5**).

DISCUSSION

In this study, we determined the number of cEPCs in T1DM patients without clinical vascular damage, of different ages and disease duration. In the first step of the study, cEPCs were measured in two separate T1DM patients' groups, either adults or pediatric, in comparison with controls of the same age range. In the second step, patients were grouped in two age groups (≥ or <20 years) and the number of cEPCs was correlated with both the age and the duration of the disease. We provide evidence that in T1DM patients without cardiovascular complications, the number of cEPCs is significantly correlated with patients' age, whereas does not depend on other clinical parameters, such as metabolic control (HbA1c), glycemic variability (MAGE), and BMI.

The number of cEPCs in adult T1DM patients turned out to be lower compared to both age-matched controls and to childhood/young T1DM patients. The latter showed very high levels of cEPCs compared to age-matched controls. The lower amount of cEPCs found in adult T1DM patients agrees with previous studies in both T1DM (20–23) and T2DM patients (9, 11–13), while the high number of cEPCs in pediatric T1DM patients is in line with what reported by Głowińska-Olszewska et al. (25) in T1DM children. Hence, the apparent contradicting data in cEPC number reported in T1DM patients in the literature could be reconciled considering the age of the patients. The number of cEPCs apparently changed also in normal subjects. In fact, although in a small number of control subjects, we found

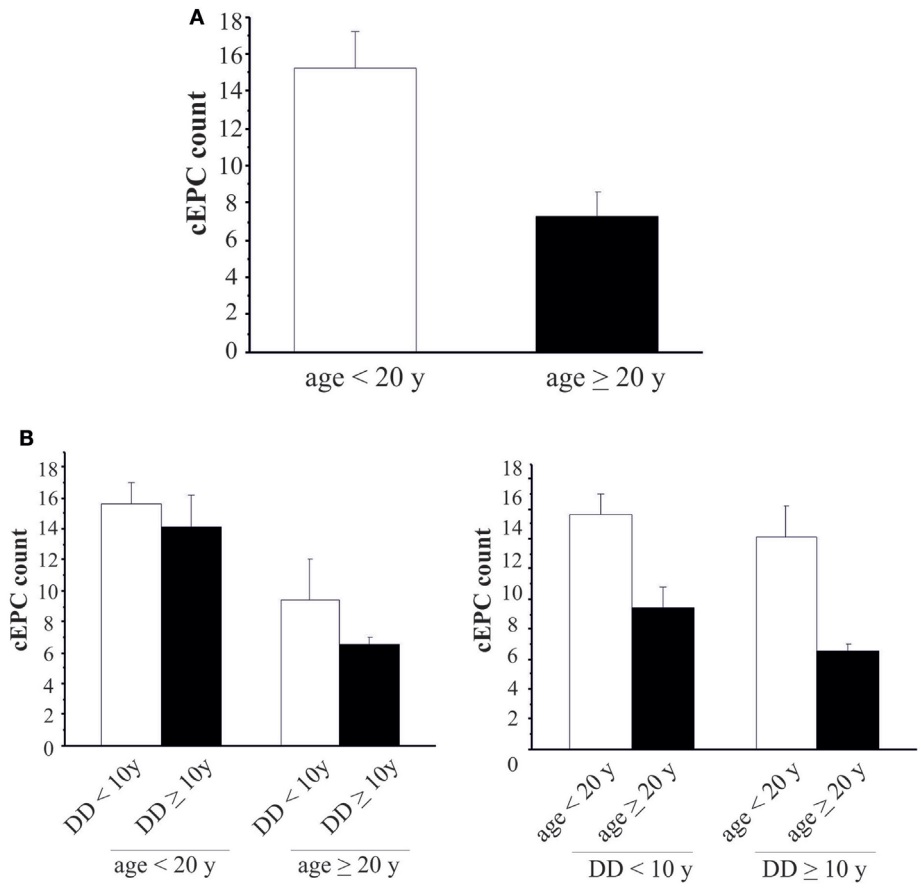

FIGURE 4 | **(A)** Circulating endothelial progenitor cell (cEPC) counts in type 1 diabetes mellitus patients of different age groups (white bar: age <20 years; black bar: age ≥20 years). Data are reported as mean ± SEM; $p = 0.004$. **(B)** Histograms summarizing cEPC levels in patients belonging to the different age and disease duration groups. Data are reported as mean ± SEM. Left histogram: $p = 0.689$ and $p = 0.418$, Mann–Whitney *U* test. Right histogram: $p = 0.153$ and $p = 0.061$, Mann–Whitney *U* test.

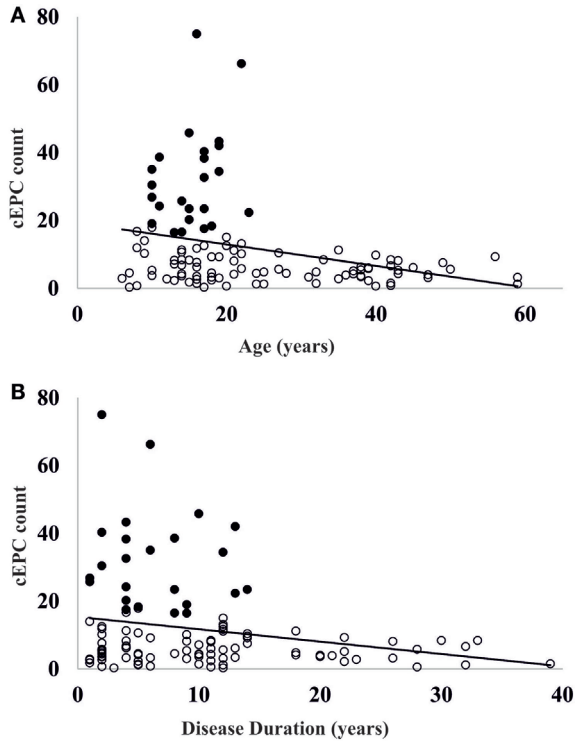

FIGURE 5 | **(A)** Scatter plot of the distribution of circulating endothelial progenitor cell (cEPC) vs age. Black circles: patients with high endothelial progenitor cell (EPC) count. **(B)** Scatter plot of cEPC vs disease duration. Black circles: patients with high EPC count.

an increase in the number of cEPCs from children to adults. Most of the data in the literature show a decrease in cEPC number in aged subjects (27, 28) and correlate this decrease with impairment in endothelial repair and onset of vascular damage (29). Our data, along with current literature, suggest the necessity to consider an age-related profile of cEPC production in physiological conditions. The progressive increase in cEPC number until young/adult age, and their progressive decrease in the elderly, could also determine differences in age-dependent cEPC production in pathological conditions.

Another aspect that must be considered when studying the number of cEPCs in different conditions is represented by the different methodologies used to analyze and quantify them. In fact, the phenotypical marker of EPCs is CD133 that is absent on mature endothelial cells, and other surface markers are $CD34^+$, $VEGFR2^+$, and $CD146^+$ (30). However, to date, there are no standardized methods to quantify and identify EPCs and the protocols used vary between studies (31). Moreover, most studies in both T2DM and T1DM patients were based on the determination of EPCs based on their growth *in vitro* (20).

Finally, the different kinds of therapies with insulin (dose and number of administration and duration of therapy) could also contribute to the different cEPC profiles in T1DM, as shown for T2DM patients (32). However, we did not find any association between the number of cEPCs and the insulin to weight ratio in the pediatric cohort analyzed in the present study.

Besides differences in cEPC number compared to controls, the most interesting result of the present study is that childhood/young (<20 years) T1DM patients have a higher number of cEPCs compared to adult (>20 years) patients. Such higher number of cEPCs is maintained also when the disease duration is longer than 10 years (**Figure 4B**, right panel). Moreover, a subpopulation of childhood/young T1DM patients, aged <20 years, whose disease lasts for less than 10 years are characterized by very high cEPC values. It is tempting to speculate whether these young patients with high cEPC levels could be protected against endothelial dysfunction. On the contrary, when the disease occurs in older age, the low levels of cEPCs could mirror a lowering of such protective effect. These still preliminary data would support the clinical observation of less incidence of late vascular complications in T1DM when the onset of the disease is in childhood respect to adult age. We wonder whether these effects could be related to a better glycemic control obtained in pediatric patients. In Hörtenhuber's prospective study, an increase in cEPC number after one year was reported in association with better glycemic control (24). In line with these results, Marfella et al. (17) showed that during percutaneous coronary intervention, an optimal periprocedural glycemia control improves myocardial salvage, by increasing cEPC number and their capability to differentiate. Notably, the same group (16) had shown that a poor glycemic control reduces EPC number in T2DM, through a mechanism that is mediated by Sirtuin expression (19).

CONCLUSION

The present study shows that a relevant association exists between the number of cEPCs and the age and duration of the disease in T1DM patients. One of the limitations of the present study is the relatively small number of patients and short follow up, as well as the lack of data on cEPC functionality, that render the data still preliminary. Nevertheless, if appropriately circumstantiated in a further study, our results might provide an additional explanation to the pathogenesis of complications in T1DM as well as to the clinical evidence of less complications in T1DM patients when the onset of the disease is in the pediatric age. Moreover, in agreement with current literature, our data suggest that maintaining a high number of cEPCs, possibly through a good glycemic control, would contribute to contain the CVD burden in T1DM.

ETHICS STATEMENT

The study was approved by Local Ethical Committee.

AUTHOR CONTRIBUTIONS

AdA, AA, and ST conceived and designed the work. MD, EL, SP, BP, LL, MC, AdA, AA, and ST acquisited, analyzed, and interpreted the data for the work. AdA, AA, and EL drafted the work or revised it critically for important intellectual content. AdA, AA, ST, EL, MD, SP, MC, BP, and LL gave final approval of the version to be published.

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Culture in Glucose-Depleted Medium Supplemented with Fatty Acid and 3,3′,5-Triiodo-L-Thyronine Facilitates Purification and Maturation of Human Pluripotent Stem Cell-Derived Cardiomyocytes

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With recent advances in stem cell technology, it is becoming efficient to differentiate human pluripotent stem cells (hPSCs) into cardiomyocytes, which can subsequently be used for myriad purposes, ranging from interrogating mechanisms of cardiovascular disease, developing novel cellular therapeutic approaches, as well as assessing the cardiac safety profile of compounds. However, the relative inability to acquire abundant pure and mature cardiomyocytes still hinders these applications. Recently, it was reported that glucose-depleted culture medium supplemented with lactate can facilitate purification of hPSC-derived cardiomyocytes. Here, we report that fatty acid as a lactate replacement has not only a similar purification effect but also improves the electrophysiological characteristics of hPSC-derived cardiomyocytes. Glucose-depleted culture medium supplemented with fatty acid and 3,3′,5-Triiodo-L-thyronine (T_3) was used during enrichment of hPSC-derived cardiomyocytes. Compared to untreated control cells, the treated cardiomyocytes exhibited enhanced action potential (AP) maximum upstroke velocity (as shown by a significant increase in dV/dt_{max}), action potential amplitude, as well as AP duration at 50% (APD_{50}) and 90% (APD_{90}) of repolarization. The treated cardiomyocytes displayed higher sensitivity to isoproterenol, more organized sarcomeric structures, and lower proliferative activity. Expression profiling showed that various ion channel and cardiac-specific genes were elevated as well. Our results suggest that the use of fatty acid and T_3 can facilitate purification and maturation of hPSC-derived cardiomyocytes.

Keywords: human pluripotent stem cell-derived cardiomyocytes, purification, maturation, fatty acid, 3,3′,5-triiodo-L-thyronine, electrophysiological characteristics

INTRODUCTION

Recent developments in stem cell technology hold promise for use in clinical applications. In the cardiovascular field, new differentiation methods now enable rapid and efficient generation of human pluripotent stem cell (hPSC)-derived cardiomyocytes (1, 2), which can subsequently be utilized as a powerful platform for cardiovascular disease modeling and moderate- to high-throughput

evaluation of drug safety profiles (3). One caveat to the accuracy of such testing is the interference from other cells (non-cardiomyocytes), so it is necessary to purify cardiomyocytes before further investigations. To date, several methods have been used for cardiomyocyte purification (4), including percoll gradient centrifugation (5), genetic modification [to generate expression of an identifier molecule driven by a cardiac-specific promoter (6, 7)], and mitochondrial dyes or antibodies against cardiac specific markers (8–11). However, these techniques all possess significant drawbacks, including low efficiency (percoll gradient centrifugation), safety concerns for therapeutic applications (genetic modification), and relatively high experimental cost (mitochondrial dyes and cardiac specific markers).

The heart's continuous mechanical function necessitates a unique metabolic profile for its constituent cardiomyocytes, which could be exploited for isolation of these cells. The two primary metabolic substrates for cardiomyocytes are glucose and fatty acid. In glycolysis, glucose is converted to pyruvate and two ATP molecules. Pyruvate is then utilized in the tricarboxylic acid (TCA) cycle in mitochondria with the generation of an additional 36 ATP molecules. In anaerobic metabolism, lactate can be converted to pyruvate by lactate dehydrogenase, which can subsequently be utilized as the substrate in the TCA cycle. In the first report of cardiomyocyte purification by using a metabolic method, Fukuda and colleagues demonstrated that glucose-depleted culture medium supplemented with lactate could be used to purify mouse and human PSC-derived cardiomyocytes (12).

However, immature cardiomyocytes use glucose as a primary substrate for generation of ATP. On the other hand, fatty acid is essential for realizing the metabolic demands of mature cardiomyocytes and represents the substrate for more than 90% of energy production in the adult heart (13). This shift from glucose to fatty acid as a primary metabolic substrate represents a key event in cardiomyocyte maturation (14); it is unknown whether replacement of a glucose-rich environment with one replete with fatty acid could help to facilitate this change. Moreover, it is known that certain molecules including 3,3′,5-Triiodo-L-thyronine (T_3), insulin-like growth factor 1 (IGF-1), and micro RNA miR-1 can promote cardiomyocyte maturation *in vitro* (14). Among these molecules, T_3 is known to positively regulate cardiac genes including *MYH6*, *TNNI3*, *NKX2.5*, *SERCA*, and *RYR2* (14–16). More importantly, T_3 can promote fatty acid oxidation (FAO) by upregulating several rate-limiting enzymes in FAO and mitochondrial biogenesis (17, 18), which may facilitate the metabolic switch from immature to mature cardiomyocytes.

Based on these data, we hypothesized that using fatty acid to replace glucose in the culture medium can both promote purification and enhance maturation of PSC-derived cardiomyocytes and that supplementation with T_3 would potentiate this process. Indeed, we found that glucose-depleted culture medium supplemented with fatty acid and T_3 can be used for purification of hPSC-derived cardiomyocytes. Moreover, compared to untreated control cells, treated cardiomyocytes exhibited a phenotype more consistent with mature cardiomyocytes, as evidenced by action potential (AP) characteristics, high sensitivity to isoproterenol, sarcomeric organization, proliferative activity, and expression levels of various ion channel and cardiac-specific genes. This highly efficient and low-cost method of hPSC-derived cardiomyocyte purification may be suitable for multiple applications where mature cardiomyocytes are required.

MATERIALS AND METHODS

Cell Culture

Human pluripotent stem cells [WA07 (H7)] from WiCell Research Institute (WI, USA), NCRM1 iPSC line from Codex BioSolutions Inc. (MD, USA), and BJ-iPSCs derived from human fibroblast cells [CRL-2522, ATCC (VA, USA)] were plated on Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Gibco, A1413202)-coated plates, and then were cultured with Essential 8 Medium (Gibco, A1517001). Experimental results and figures in this paper were obtained mainly using hESCs (WA07) and confirmed by other hiPSCs. The differentiation protocol was modified based on the published protocols (1, 2). Briefly, hPSC were treated with small molecule CHIR99021 (Tocris, 4423, final concentration 10 μM) in the RPMI-BSA medium [RPMI 1640 Medium (HyClone, SH30027.01) supplemented with 213 μg/ml AA2P (L-ascorbic acid 2-phosphate magnesium) (A8960, Sigma) and 0.1% bovine serum albumin (BSA) (A1470, Sigma)] for 24 h, then were incubated with RPMI-BSA medium for 48 h. On differentiation day 4, cells were treated with the small molecule IWP2 (Tocris, 3533, final concentration 5 μM) in RPMI-BSA medium. After 48 h, media were changed to RPMI-BSA medium. Then, RPMI 1640 Medium supplemented with 3% KnockOut Serum Replacement (Gibco, 10828-028, the routine medium) was used to culture the cardiomyocytes in the following experiments. In general, contracting cardiomyocytes could be observed on differentiation day 9–11.

Metabolic Selection

According to the previous report (12), lactate medium was prepared as DMEM Medium (No Glucose) (Gibco, 11966-025) supplemented with Sodium DL-lactate (Sigma, L4263, final concentration 4 mM). Fatty acid medium was prepared as DMEM Medium (No Glucose) supplemented with 0.1% BSA (Sigma, A1470) and 1× Linoleic Acid-Oleic Acid-Albumin (Sigma, L9655). Fatty acid + T_3 medium was fatty acid medium supplemented with T_3 (Acros Organics, 437260010, final concentration 10 nM). Cells were treated with metabolic selection medium (lactate, fatty acid and fatty acid + T_3) for purification and cultured with routine medium as controls. The medium was changed every 2 days and the whole selection process lasted no longer than 9 days.

Cell Viability Test

Human induced pluripotent stem (iPS) cells, human embryonic stem (ES) cells, mouse ES cells, mouse neonatal cardiomyocytes, and mouse HL-1 cells were exposed to metabolic selection medium (lactate and fatty acid) and glucose-free DMEM medium. At each time point, cells were trypsinized using 0.25% Trypsin-EDTA (Gibco, 25200-056). After serum neutralization, the trypsinized cells were centrifuged for 4 min at 1,000 rpm,

resuspended in 100 μl phosphate-buffered saline (PBS), stained with 0.4% Trypan Blue Solution (Gibco, 15250-061), and counted using a hemocytometer. The cell viability rate equals the number of live cells/the cell number at the beginning of purification.

Intracellular Staining for Fluorescence-Activated Cell Sorting (FACS) Using Troponin T Cardiac Isoform Antibody

Cardiomyocytes were dissociated using 0.05% Trypsin-EDTA and then fixed with 4% paraformaldehyde (Electron Microscopy Sciences, 15714-S) for 20 min at room temperature. Cells were permeabilized and blocked with 1× PBS supplemented with 0.15% Triton X-100 (Sigma, T9284) and 10% goat serum (Millipore, S26-LITER) for 15 min at room temperature. Then cells were washed twice with 1× PBS supplemented with 0.2% Tween 20 (Bio-Rad, 1706531) and 0.1% BSA. Subsequently, cells were stained with Troponin T Cardiac Isoform antibody (Thermo Fisher, MA5-12960) at room temperature for 1 h. After washing with 1× PBS containing 0.2% Tween 20 and 0.1% BSA, cells were incubated with the Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Thermo Fisher, A-11001) at room temperature for 1 h. These cells were sorted using the LSRII analyzer (BD). FACS results were analyzed using FlowJo software.

Immunofluorescence Staining

Cardiomyocytes were dissociated using 0.05% Trypsin-EDTA and plated onto microscope cover slips (Fisher Scientific, 051115-9) prior to purification by metabolic selection medium. After purification, cells were fixed with 4% paraformaldehyde at room temperature for 20 min and washed three times with 1× PBS. Cells were then permeabilized with PBS containing 0.25% Triton X-100 at room temperature for 10 min. After incubating in the blocking buffer (1× PBS with 10% goat serum), cells were stained with different primary antibodies at 4°C overnight (Troponin T Cardiac Isoform antibody, Thermo Fisher, MA5-12960; Cardiac Troponin I antibody, Abcam, ab47003; Anti-α-Actinin (Sarcomeric) antibody, Sigma, A7811; Anti-IK1 antibody, Santa Cruz, sc-365265; Anti-SERCA2 antibody, Santa Cruz, sc-73022; Anti-CPTI antibody, Santa Cruz, sc-393070). Cells were washed three times with PBS containing 0.1% Triton X-100, then incubated with the Alexa Fluor 488 goat anti-mouse or Alexa Fluor 555 goat anti-rabbit IgG secondary antibodies at room temperature for 1 h. Nuclei were labeled with DAPI (4′,6-diamidino-2-phenylindole, 1 μg/ml) for 5 min. Cells were observed using AxioObserver Inverted SK-2 microscopy (Zeiss). Image analysis was performed with ImageJ software. Quantification of the FITC fluorescence intensity for each condition was performed using Zen 2.3 lite software (Zeiss). For measurement of sarcomere length, we followed the protocol from the previous report (14). Briefly, myofibrils with at least five continuous and organized α-Actinin-positive bands were selected and measured. Length measurement was performed using Zen 2.3 lite software (Zeiss).

AP Recording

All electrophysiological recordings were conducted at room temperature using an Axonmulticlamp 700B Amplifier and a pClamp system (versions 10.2, Axon Instruments). For spontaneous AP current clamp recordings, pipettes were filled with a solution containing (in mmol/l): KCl 135, $MgCl_2$ 1, EGTA 10, HEPES 10, and glucose 5, pH 7.2 with KOH. The bath solution contained (in mmol/l): NaCl 136, KCl 4, $CaCl_2$ 1, $MgCl_2$ 2, HEPES 10, and glucose 10, pH 7.4 with NaOH. The AP maximum upstroke velocity (dV/dt_{max}), the maximum negative potentials, action potential amplitudes (APAs), as well as AP durations at 50% (APD_{50}) and 90% (APD_{90}) of repolarization were measured. To avoid the influences of the spontaneous beating rates on the APD, the corrected APD (cAPD) by heart rates [APD/square root of the cycle length between two spontaneous APs (RR)] was used for average and compared between different groups (19).

Calcium Imaging

Intracellular Ca^{2+} transients were studied by using the IonOptix microfluorimetry system (IonOptix Inc., MA, USA). According to the standard protocol, cardiomyocytes derived from hPSCs were loaded with Fluo-8/AM (Molecular Probes) in Tyrode buffer for 60 min at 37°C. Then, cardiomyocytes were perfused with Tyrode buffer supplemented with 1.8 mM $CaCl_2$, 5.5 mM D-glucose, and 0.5 m Mprobenecid and maintained at 35°C–37°C. Cells were field stimulated at 0.5 Hz using a MyoPacer Field Stimulator (IonOptix Inc., MA, USA). Intracellular calcium transients were analyzed with IonWizard software. The Ca^{2+} transient peak amplitude was denoted F/F_0, in which F_0 presented the fluorescence intensity at the onset of the experiment. SR calcium content of store was evaluated following rapid caffeine application (10 mM). To evaluate the effect of isoproterenol administration, intracellular Ca^{2+} transients were measured before and after isoproterenol (100 nM) application.

Quantitative PCR

Total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, 74104) prior to the treatment with DNase I (Thermo Fisher, EN0525) for 30 min. mRNA was reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad, 1708841). Quantitative PCR was performed using a Mastercycler RealPlex^2 (Eppendorf) with SsoFast EvaGreen Supermix (Bio-Rad, 1725200). The quantitative PCR primers designed from qPrimerDepot (20) are as followed (from 5′ to 3′):

KCNA4-Forward: CAGCCAAAATCATGCAGAAG;
KCNA4-Reverse: GATCATTCTTCCCCTCCTCC;
KCND3-Forward: AAACAATCACAGGGACTGGC;
KCND3-Reverse: ACACCATTGTCACCATGACC;
KCNH2-Forward: TCCTTCTCCATCACCACCTC;
KCNH2-Reverse: AAATCGCCTTCTACCGGAAA;
KCNQ1-Forward: ACAAAGTACTGCATGCGTCG;
KCNQ1-Reverse: CATGAGAACCAACAGCTTCG;
KCNN4-Forward: GGACCTCTTTGGCATGAAAG;
KCNN4-Reverse: CATGCAGAGATGCTGTGGTT;
CACNA1C-Forward: TAGGCATTGGGGTGAAAGAG;
CACNA1C-Reverse: GAAGATGATTCCAACGCCAC;
SCN5A-Forward: GAGGGCAATGATCTTGAAGG;
SCN5A-Reverse: TCAACACACTCTTCATGGCG;
TNNI3-Forward: GCCCACCTCAAGCAGGTG;

TNNI3-Reverse: TTGCGCCAGTCTCCCACCTC;
TNNI1-Forward: ACAAGGTGCTGTCTCACTGC;
TNNI1-Reverse: CTCTTCAGCAAGAGTTTGCG;
MYH6-Forward: CAAGTTGGAAGACGAGTGCT;
MYH6-Reverse: ATGGGCCTCTTGTAGAGCTT;
MYH7-Forward: GGGCAACAGGAAAGTTGGC;
MYH7-Reverse: ACGGTGGTCTCTCCTTGGG;
CPT1A-Forward: GCCTCGTATGTGAGGCAAA;
CPT1A-Reverse: CCCATTCGTAGCCTTTGGTA;
CPT1B-Forward: GCTTGATTTCTTCACGGTCC;
CPT1B-Reverse: CCTCTCATGGTGAACAGCAA;
ACOX1-Forward: ATGCCCAAGTGAAGATCCAG;
ACOX1-Reverse: GAGGTGGCTGTCAGGAAAAG;
PPARGC1A-Forward: CTGCTAGCAAGTTTGCCTCA;
PPARGC1A-Reverse: GCTTTCTGGGTGGACTCAAG;
NRF1-Forward: GCCACTGCATGTGCTTCTAT;
NRF1-Reverse: GTCGCAGTCTCCACGGC.

BrdU Assay

The BrdU assay was performed using a Bromo-2′-deoxy-uridine Labeling and Detection Kit I (Roche, 11296736001). We followed the instructions provided by the manufacturer prior to double-staining with cardiac Troponin I antibody (Abcam, ab47003). Cells were incubated with the primary antibody for 1 h at 37°C, then washed three times with PBS containing 0.1% Triton X-100. Cells were incubated with the Alexa Fluor 488 goat anti-rabbit IgG secondary antibody and the Alexa Fluor 555 goat anti-mouse IgG secondary antibody at room temperature for 1 h. Images were captured using AxioObserver Inverted SK-2 microscopy (Zeiss) and analyzed using ImageJ software.

Statistics

Values are expressed as mean ± SD. Statistical significances were evaluated using one-way ANOVA with Bonferroni correction. $P < 0.05$ was considered statistically significant. The statistical significances between experimental groups in each figure were shown in Table S1 in Supplementary Material.

RESULTS

A protocol for human iPS cell differentiation to a cardiac lineage was modified from the method previously described, allowing acquisition of contracting cardiomyocytes as early as on differentiation day 9 (1, 2). Differentiated cells would then be subjected to an 8-day metabolic selection process. **Figure 1A** represents a schematic of this purification and maturation treatment.

Glucose-Depleted Culture Medium Supplemented with Fatty Acid Can Enrich for hPSC-Derived Cardiomyocytes

First, we assessed the relative abilities of multiple cell lines to tolerate various culture conditions. Human iPS cells, human ES cells, murine ES cells, murine neonatal cardiomyocytes, and murine

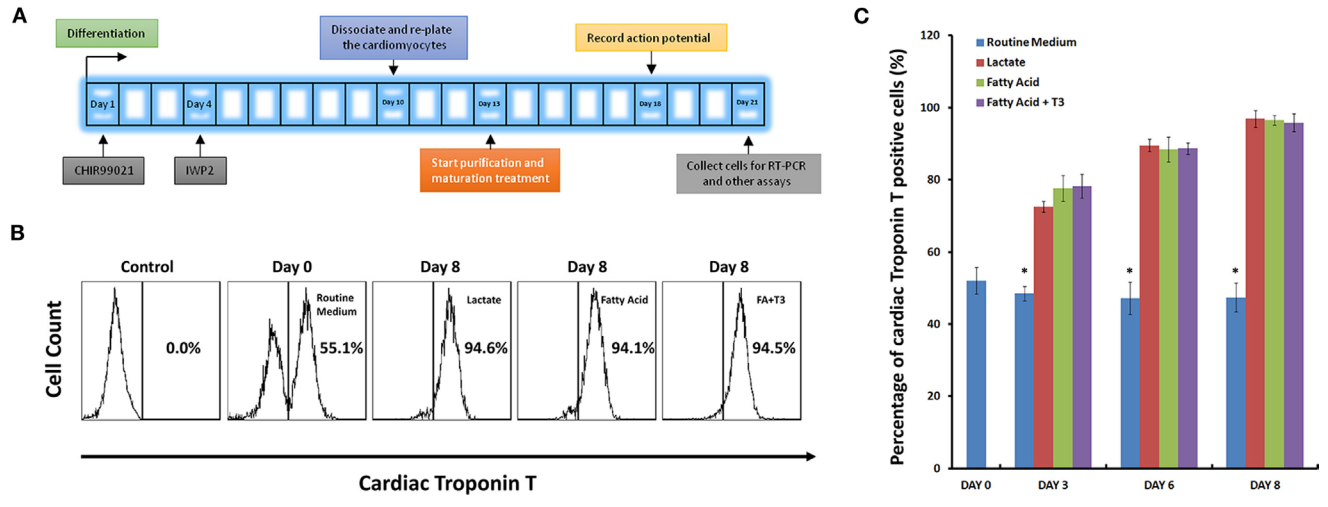

FIGURE 1 | High purity of cardiomyocytes can be obtained under glucose-depleted and fatty acid (with or without T_3)-supplemented conditions. **(A)** The schematic for purification and maturation using metabolic selection. Glycogen synthase kinase 3 inhibitor CHIR99021 and Wnt pathway inhibitor IWP2 (gray boxes) were added to the differentiation medium on differentiation days 1 and 4, respectively. On differentiation day 10, cells were dissociated and plated on glass cover slips. After 3 days recovery, cells were cultured with metabolic selection medium (No-glucose DMEM medium supplemented with lactate, fatty acid, and fatty acid + T_3). On differentiation day 18, cells were collected for patch clamping experiments. On differentiation day 21, cells were collected for other experiments. **(B)** Representative fluorescence-activated cell sorting (FACS) analyses of cardiac Troponin T expression in the human pluripotent stem cell (hPSC)-derived cells on day 0 with routine culture medium, and on day 8 with metabolic selection medium. In the control group, the isotype control (mouse IgG) was used instead of the primary antibody. **(C)** Time courses of selection efficiency using the lactate-supplemented condition (red), the fatty acid-supplemented condition (green), and the fatty acid with T_3-supplemented condition (purple); cells cultured with routine medium (blue) were set up as the control groups ($n = 3$). The asterisk means each test group is significantly different from controls, $P < 0.01$.

HL-1 cells were cultured in non-glucose medium, non-glucose medium supplemented with lactate, and non-glucose medium supplemented with fatty acid (Figure S1 in Supplementary Material). With the exception of murine neonatal cardiomyocytes, no cell type could survive without glucose beyond 5 days. Culturing in lactate—compared to fatty acid-supplemented non-glucose media exerted similar effects on human iPS cells, human ES cells, murine ES cells, and murine neonatal cardiomyocytes. Interestingly, murine HL-1 cells appeared more adaptive to the lactate-supplemented glucose-free medium, likely as a consequence of the general preference of neoplastic cells for lactate as a metabolic substrate (21). These data suggested a relative selection bias for cardiomyocytes as compared to other cell types when cultured in glucose-free conditions supplemented with either lactate or fatty acid.

Next, we compared the purification efficiency of several methods of metabolic selection. hPSC-derived cardiomyocytes were cultured in glucose-free medium supplemented with lactate, fatty acid, or a combination of fatty acid and T_3. After 8 days in these conditions, cells were dissociated, stained with anti-cardiac Troponin T (*TNNT2*) antibody, and analyzed by FACS. As defined by positivity for *TNNT2*, each of these three media conditions demonstrated similar purification efficiency of hPSC-derived cardiomyocytes (up to 95%, **Figures 1B,C**; Figure S2 in Supplementary Material).

Fatty Acid and T_3-Treated Cardiomyocytes Exhibited Mature AP Morphology

Action potential morphology is one of the basic indices of electrophysiological maturity of cardiomyocytes, while mature hPSC-derived cardiomyocytes should demonstrate similar AP morphology when compared to adult cardiomyocytes (14, 16). We cultured hPSC-derived cardiomyocytes in glucose-containing medium (Routine Medium), as well as several glucose-depleted conditions [medium supplemented with lactate (Lactate), medium supplemented with fatty acid (Fatty Acid), and medium supplemented with fatty acid and T_3 (Fatty acid + T_3)]. After culturing for 5 days in these conditions, AP morphology was assessed with whole-cell patch clamping. Compared to cardiomyocytes cultured in the routine medium, fatty acid and T_3-treated cardiomyocytes exhibited AP morphology more consistent with adult cardiomyocytes (**Figure 2**). Notably, the combination of fatty acid and T_3 was the only culture condition that could improve all the characteristics of AP, including dV/dt_{max}, the maximum negative potential, APA, AP duration at 50% of repolarization (APD_{50}), and the corrected AP duration at 90% of repolarization ($cAPD_{90}$). Likewise, compared to the routine culture condition, culturing in lactate or fatty acid conditions could also make the maximum negative potential more hyperpolarized and prolong the AP duration, but had no significant (NS) effect on dV/dt_{max} or APA.

Fatty Acid and T_3-Treated Cardiomyocytes Demonstrated Improved Response to Caffeine and Isoproterenol

We performed calcium imaging with a microfluorimetry system to assess effects of the three culture conditions (routine medium, lactate, and fatty acid + T_3) on calcium handling. There were NS differences for the Ca^{2+} transient peak amplitude and decay time constant between different culture conditions while pacing at 0.5 Hz (**Figures 3A–C**). Caffeine was then administered to evaluate the sarcoplasmic reticulum Ca^{2+} content of the store (22). There were NS differences for the Ca^{2+} transient peak amplitude after caffeine application. However, the tau of fatty acid and T_3-treated cardiomyocytes was significantly reduced compared to cardiomyocytes from the other culture conditions (**Figure 3C**), which was suggestive that fatty acid and T_3 treatment could improve the function of sodium–calcium exchanger. Isoproterenol was administered to evaluate the effects of β_1 and β_2 agonism. After isoproterenol application, the peak amplitude of Ca^{2+} transients and beating rates were increased in cardiomyocytes from each of the three culture conditions. However, the response to isoproterenol was significantly greater in fatty acid and T_3-treated cardiomyocytes (**Figures 3D–F**).

Fatty Acid and T_3 Treatment Associated With Increased Expression of Major Ion Channel and Cardiac-Specific Genes

As a hormone, T_3 binds nuclear receptors and regulates the transcription of various genes (23, 24). We measured the expression of major ion channel and cardiac genes in each of the three culture conditions compared to the routine medium condition. We noted significant upregulation of genes encoding for the major cardiac potassium channel subunits associated with the fatty acid and T_3 treatment: *KCND3* and *KCNQ1* expression, which are known to be more pronounced in canine adult (as compared to neonatal) ventricular tissue (25), were increased 7.1- and 1.5-fold, respectively. *KCNJ2*, which encodes for the pore of the inward rectifier K^+ channel (26), a key regulator of phase 3 and known to be upregulated in cardiac development (27), increased 4.9-fold. *KCNH2* and *KCNN4* increased 1.4- and 3.8-fold, respectively. The exception was *KCNA4*, which appeared to be downregulated (**Figure 4A**). *SCN5A*, which encodes for Nav1.5 and is responsible for phase 0 of the cardiac AP, was upregulated 1.3-fold with culture in fatty acid and T_3, while the expression of *CACNA1C* (encoding for Cav1.2 of the voltage dependent L-type calcium channel) was downregulated (**Figure 4A**). Overall, these results are consistent with previously published data in murine, rat, and canine cardiomyocytes and suggest the emergence of a more mature phenotype associated with fatty acid and T_3 treatment.

We next examined relative expression levels of cardiac-specific genes (**Figure 4B**). We found that the expression of *TNNI3*, encoding for the cardiac biomarker troponin I (14), was significantly increased after fatty acid + T_3 treatment (6.93-fold versus culture in routine medium). This is consistent with data suggesting that T_3 can increase expression of *TNNI3* in the rat heart (28). *ATP2A2* is also known to be upregulated with cardiomyocyte maturation (14); its product, SERCA2, plays a pivotal role in excitation-contraction coupling in mature cardiomyocytes (29). Unsurprisingly, *ATP2A2* exhibited a 5.4-fold increase in expression when cardiomyocytes were cultured in fatty acid and T_3 compared to cardiomyocytes in the routine culture medium,

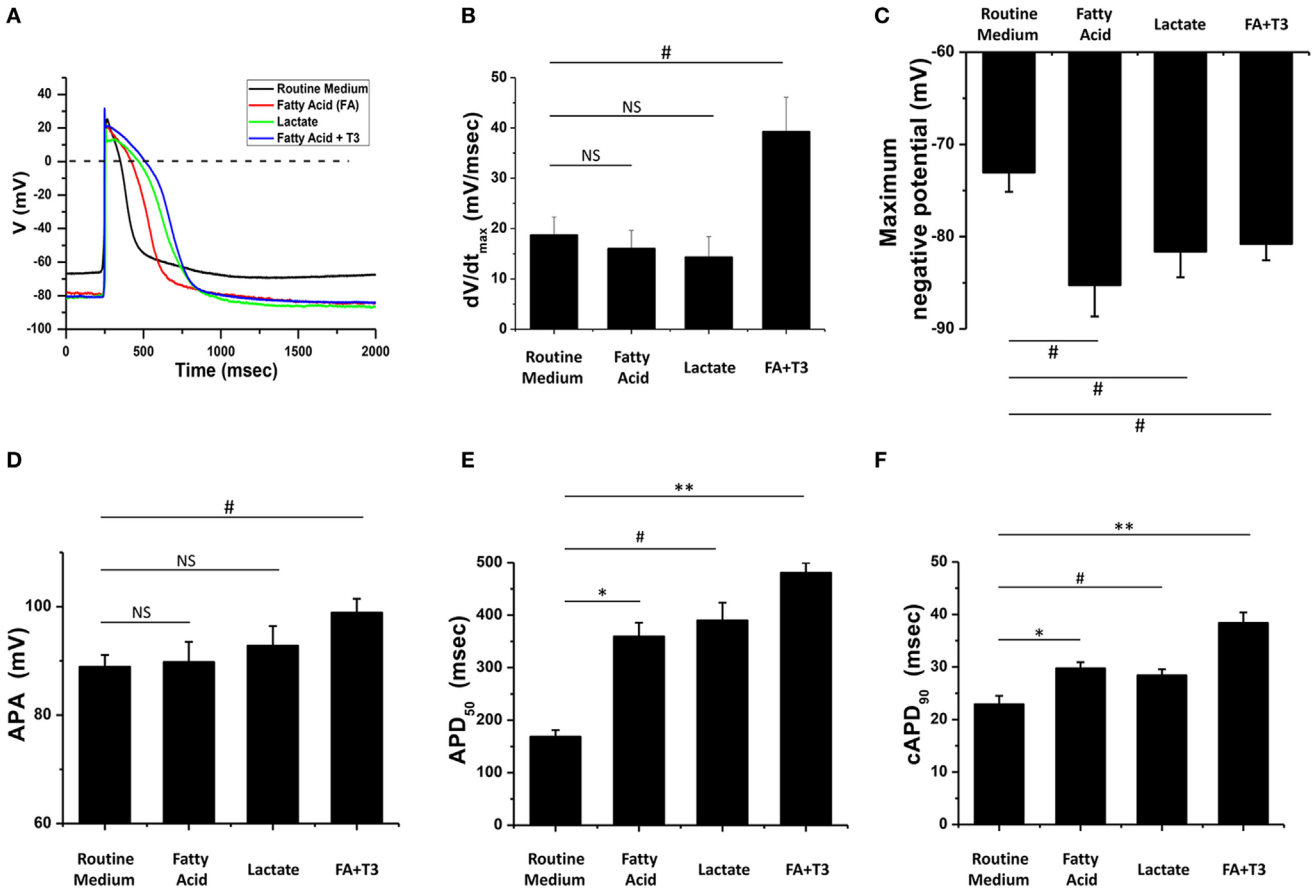

FIGURE 2 | Fatty acid and T_3-treated cardiomyocytes exhibited more mature action potential (AP) morphology. **(A)** Representative AP traces of cardiomyocytes cultured with metabolic selection medium and routine medium. **(B–F)** AP indices of dV/dt_{max}, the maximum negative potential, action potential amplitude (APA), AP duration at 50% of repolarization (APD_{50}), and the corrected AP duration at 90% of repolarization ($cAPD_{90}$) from cardiomyocytes cultured with metabolic selection medium and routine medium (routine medium, $n = 20$ cells; fatty acid, $n = 18$ cells; lactate, $n = 17$ cells; fatty acid + T_3, $n = 20$ cells). Only the combination of fatty acid + T_3 treatment significantly improved all indices. NS, no significant difference; $^{\#}P < 0.05$; $^{*}P < 0.01$; $^{**}P < 0.001$.

which is suggestive of improved calcium-handling and consistent with the microfluorimetry data. Interestingly, *MYH6* (encoding for the α isoform of the myosin heavy chain) was upregulated and *MYH7* (encoding for the β isoform of myosin heavy chain) was downregulated, which is discrepant with protein levels in human hearts, where the β isoform predominates over the α isoform (30, 31). However, our results were similar to the previous reports (14, 32). This might be one of the transcriptional regulation effects of T_3. Unsurprisingly, the genes encoding for carnitine palmitoyltransferase I and Acyl-CoA oxidase (*CPT1A*, *CPT1B*, and *ACOX1*), the rate-limiting enzymes for FAO, were all upregulated with culture in fatty acid + T_3 (**Figure 4C**). *PPARGC1A*, which encodes the protein involved in mitochondrial biogenesis, demonstrated enhanced expression after fatty acid + T_3 treatment as well.

To confirm the results from quantitative real-time PCR (qPCR) assays, IK1, SERCA2, and CPTI were selected to perform immunostaining in cardiomyocytes from each condition. The microscopic observations were consistent with the results from qPCR (Figures S3–S5 in Supplementary Material).

Fatty Acid and T_3 Enhance Sarcomere Formation and Decrease Proliferative Activity in hPSC-Derived Cardiomyocytes

The sarcomeric structure is one of the hallmarks of mature cardiomyocytes (14). Following the criteria from previous reports (33, 34), we estimated the proportion of cardiomyocytes with organized sarcomeres in cells cultured in each of the three conditions. We found that a higher proportion of fatty acid and T_3-treated cardiomyocytes displayed organized sarcomeres compared to the routine medium condition (**Figures 5A,B**). The sarcomere length of cardiomyocytes from each culture condition was also measured. Fatty acid and T_3-treated cardiomyocytes exhibited increased sarcomere length compared to controls (Figure S6 in Supplementary Material). It has been reported that exposure to T_3 can decrease the proliferation rate of iPSC-derived cardiomyocytes (14). Then, we utilized a BrdU-based cell proliferation assay to evaluate hPSC-derived cardiomyocytes cultured in each of the three conditions. We demonstrated that the proportion of BrdU positive cardiomyocytes was significantly decreased in both

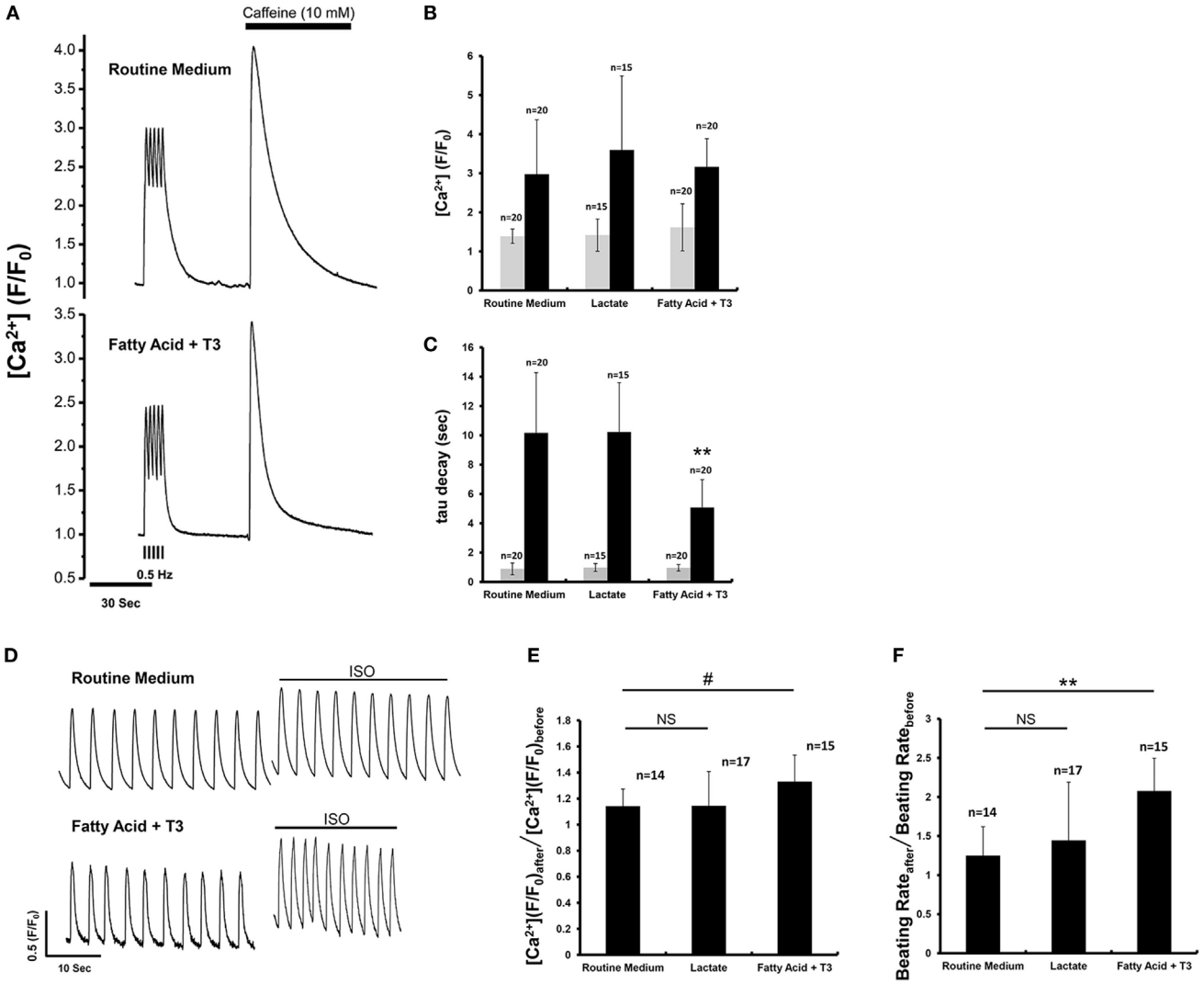

FIGURE 3 | Fatty acid and T_3-treated cardiomyocytes demonstrated improved calcium-handling in response to caffeine and isoproterenol. **(A)** The representative traces of calcium transient before and after caffeine application. **(B,C)** Calcium transients peak amplitude and decay time constant (tau) before (gray boxes) and after (black boxes) caffeine administration from cardiomyocytes maintained in the various culture conditions. Before caffeine administration, calcium transient peak amplitude and tau were recorded from the 0.5 Hz-paced cardiomyocytes. The double asterisks mean tau values of fatty acid and T_3-treated cardiomyocytes were significantly different from the values of other two experimental groups. **(D)** The representative traces of calcium transient before and after isoproterenol application. **(E)** The ratio of calcium transient peak amplitude after isoproterenol application to that before isoproterenol application. Fatty acid and T_3-treated cardiomyocytes demonstrated a greater response to isoproterenol than the other groups. **(F)** The ratio of beating rates after isoproterenol application to that before isoproterenol application. *n* indicates tested cells. $^{\#}P < 0.05$; $^{**}P < 0.001$.

fatty acid + T_3 (0.032 ± 0.009 versus 0.108 ± 0.032 in control cells, $P < 0.001$) and lactate (0.033 ± 0.027 versus 0.108 ± 0.032 in control cells, $P < 0.001$) culture conditions (**Figures 5C,D**). This is suggestive that metabolic selection with fatty acid + T_3 in hPSC-derived cardiomyocytes may improve the development of sarcomeric structure and lead to lower proliferative activity of cardiomyocytes, which were observed in adult cardiomyocytes.

DISCUSSION

Applications for hPSCs are rapidly expanding, especially in the realm of cardiovascular research. hPSC-derived cardiomyocytes are used to probe mechanisms of cardiovascular pathology, assess drug safety, and be a possible therapeutic tool. This last use is regarded by many as the "holy grail" of cardiovascular therapeutics. As the most frequent cause of death in the developed world, ischemic heart disease is incited by an initial loss of cardiomyocytes during acute myocardial infarction, which serves subsequently as an impetus for myocardial remodeling. The ability to replace lost or injured cardiomyocytes with mechanically and electrically healthy counterparts, therefore, represents an effective treatment for many forms of cardiovascular diseases: hPSCs represent an unlimited source for such healthy cardiomyocytes.

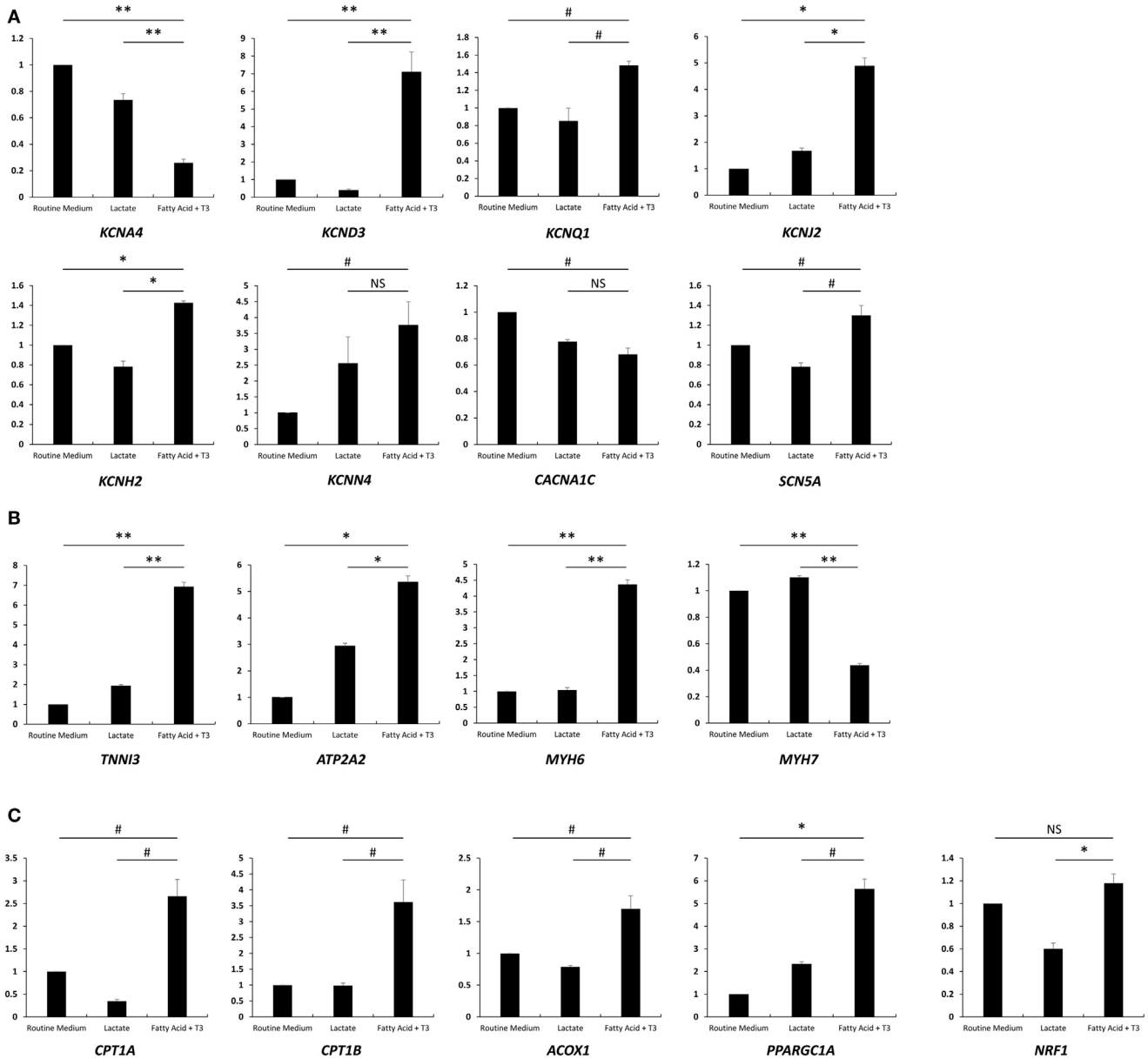

FIGURE 4 | Fatty acid and T_3 treatment is associated with increased expression of major ion channel and cardiac-specific genes. Total mRNA was extracted from cells cultured in the lactate-supplemented condition, the fatty acid + T_3-supplemented condition, as well as routine medium ($n = 3$). Expression of major ion channel genes, cardiac genes, and fatty acid oxidation-related genes are shown in **(A–C)**, respectively. NS, no significant difference; $^{\#}P < 0.05$; $^{*}P < 0.01$; $^{**}P < 0.001$.

There are several major hurdles that must first be overcome before the myriad potential uses of hPSC-derived cardiomyocytes can be realized. One of these is the purification of generated cardiomyocytes. Despite recent advancements in the efficiency of cardiac differentiation of hPSCs, the resulting cell population remains a heterogeneous pool of cardiomyocytes and other cells (35). Since cardiomyocytes exhibit marked phenotypic differences from other cell-types, the validity of conclusions drawn from their investigational use and the safety of their therapeutic use is predicated upon their purification from non-cardiomyocytes.

According to the unique metabolic features of cardiomyocytes, it is logical to make an assumption that cardiomyocytes, but not non-cardiomyocytes, are able to survive by taking advantage of certain metabolic substrates under metabolic selection. Fukuda and his colleagues first reported that PSC-derived cardiomyocytes could be purified by a glucose-depleted medium supplemented with lactate. Their solid work clarified that metabolic selection was a high-efficiency way for the enrichment of cardiomyocytes. In their research, the distinct metabolic differences between cardiomyocytes and ES cells in transcriptome were fully demonstrated. Genes involved in the TCA cycle exhibited higher

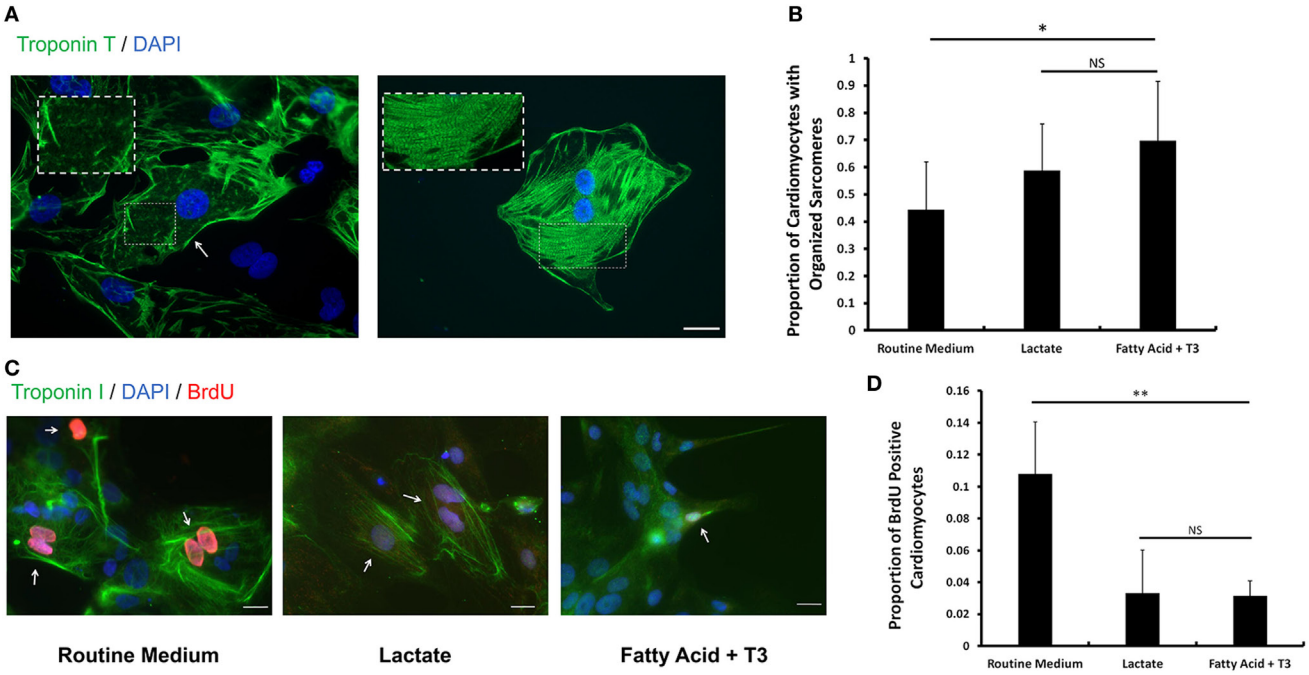

FIGURE 5 | Fatty acid and T_3 treatment enhances sarcomere formation and decreases proliferative activity in human pluripotent stem cell (hPSC)-derived cardiomyocytes. **(A)** Representative photomicrographs showing cardiac Troponin T (green) in hPSC-derived cardiomyocytes with unorganized (left panel) and organized (right panel) sarcomeres. Magnification of the sarcomeres is shown in the white dash line boxes. Nuclei are stained with DAPI (blue). The white arrow indicates the cardiomyocyte with undeveloped sarcomeres cultured in the routine medium. The scale bar is 200 μm. **(B)** The proportion of cardiomyocytes with organized sarcomeres from the lactate-supplemented condition, the fatty acid + T_3-supplemented condition and the routine culture condition. $n > 1300$ cells in each group. $^{\#}P < 0.05$; $^{*}P < 0.01$. **(C)** Fatty acid and T_3-treated cardiomyocytes demonstrate lower proliferative activity. Bromo-2′-deoxy-uridine labeling was performed. Cells were co-stained with cardiac Troponin I antibody (green), DAPI (blue), and BrdU antibody (red). Representative images were taken from cells cultured in control medium, in fatty acid + T_3 medium, and in lactate medium. The white arrows denote BrdU-positive cardiomyocyte nuclei. The scale bar is 20 μm. **(D)** Fatty acid + T_3 treatment and lactate treatment are associated with a significantly lower percentage of BrdU-positive cardiomyocytes than the control condition. Approximately 8,000 cells were counted in each group. NS, no significant difference; $^{**}P < 0.001$.

expression levels in cardiomyocytes than in ES cells. They also proved that cardiomyocytes could use lactate as an alternative energy substrate more efficiently than non-cardiomyocytes, so only cardiomyocytes could obtain essential ATPs and survive in the glucose-depleted culture medium supplemented with lactate. As we know, glucose and fatty acid are two major metabolic substrates for TCA cycle. Glucose can be converted into pyruvate then pyruvate turns into Acetyl CoA; fatty acid can also be converted into Acetyl CoA through several steps. In our study, fatty acid, an alternative metabolic substrate of cardiomyocytes, was selected as a replacement of glucose in the purification culture medium. Our data clearly illustrated that glucose-depleted medium supplemented with fatty acid could efficiently enrich hPSC-derived cardiomyocytes. Therefore, it is reasonable to deduce the mechanism of purification effect by using the glucose-depleted culture medium supplemented with fatty acid: like lactate, fatty acid can be used by cardiomyocytes with high efficiency to produce enough ATPs, while non-cardiomyocytes (especially stem cells) could not utilize fatty acid efficiently. So only cardiomyocytes could survive under this condition. More importantly, most hPSC-derived cardiomyocytes could survive under 8-day metabolic selections (the survival rates of cardiomyocytes under lactate or fatty acid + T_3 conditions were as high as 96%, data not shown). It is important to note that free fatty acid has been reported as being toxic to cardiomyocytes (36, 37). This property can be ameliorated by supplementing the culture medium with BSA, which is reported to bind the free fatty acid, allowing for a complex that can be transported to the intracellular space (38, 39). For this reason, we supplemented our selection medium with BSA; we attribute the survival of cardiomyocytes in our study to this medium change. With this in mind, purification by metabolic selection should be carefully adopted for hPSC-derived cardiomyocytes from patients with endocrine or metabolic disorders, because these cardiomyocytes probably could not suffer from the metabolic stress, which leads to failure of cardiomyocyte enrichment.

Human pluripotent stem cell-derived cardiomyocytes spontaneously depolarize and contract. This feature is conferred by a prominent I_f and suggestive of an immature electrical phenotype. In fact, previous studies suggest that hPSC-derived cardiomyocytes more closely resemble fetal cardiomyocytes as compared to their adult counterparts (40). Despite the relative ability of hPSC-derived cardiomyocytes to detect delayed repolarization elicited by drugs with known effects on hERG or the clinical ECG

(41–50), mature hPSC-derived cardiomyocytes are more ideal for probing mechanisms of adult-onset cardiovascular disease and for studies of pharmacological agents to be utilized in an adult population. As the shift from glucose to fatty acid as a primary metabolic substrate represents a key event in cardiomyocyte maturation (14), we hypothesized that this intervention could facilitate maturation. Moreover, since T_3 can promote cardiomyocyte maturation (14), positively regulate cardiac genes (*MYH6*, *TNNI3*, *NKX2.5*, *SERCA*, and *RYR2*) (14–16) and upregulate several rate-limiting enzymes in FAO and mitochondrial biogenesis (17, 18), we hypothesized that this hormone could potentiate the maturation process. Indeed, compared to untreated control cells, treated cardiomyocytes exhibited a phenotype more consistent with mature cardiomyocytes, as evidenced by AP characteristics, sensitivity to isoproterenol, sarcomeric organization, proliferative activity, and expression levels of various ion channel and cardiac-specific genes.

dV/dt_{max}, which reflects the rate of depolarization, is associated with the physiological and functional performance of I_{Na} (51). T_3 might play a critical role in the enhancement of depolarization in cardiomyocytes. In addition to T_3, we tested several other compounds with the reported ability to promote cardiomyocyte maturation (including phenylephrine, IGF-1, and WY-14643) in our culture system (52, 53). We found that only the combination of fatty acid and T_3 could significantly improve the AP morphology of hPSC-derived cardiomyocytes, while other chemicals exhibited no differences compared to control culture conditions (data not shown).

In conclusion, we have developed an efficient and inexpensive method of purification and promoting maturation of hPSC-derived cardiomyocytes using glucose-depleted culture medium supplemented with fatty acid and T_3. This method may be suitable for multiple applications where mature cardiomyocytes are required.

AUTHOR CONTRIBUTIONS

BL, XL, MS, and LB had substantial contributions to the design of the paper; BL, XL, MS, EW, YL, and LB contributed to the conception of the experiments and analyses of data; JL, XS and MD provided critical suggestions to improve the paper; BL, XL, MS, JL, and LB wrote the manuscript. All authors (BL, XL, MS, EW, YL, JL, XS, MD and LB) have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fendo.2017.00253/full#supplementary-material.

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REVIEW
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Cerebral Pathology and Cognition in Diabetes: The Merits of Multiparametric Neuroimaging

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Type 2 diabetes mellitus is associated with accelerated cognitive decline and various cerebral abnormalities visible on MRI. The exact pathophysiological mechanisms underlying cognitive decline in diabetes still remain to be elucidated. In addition to conventional images, MRI offers a versatile set of novel contrasts, including blood perfusion, neuronal function, white matter microstructure, and metabolic function. These more-advanced multiparametric MRI contrasts and the pertaining parameters are able to reveal abnormalities in type 2 diabetes, which may be related to cognitive decline. To further elucidate the nature of the link between diabetes, cognitive decline, and brain abnormalities, and changes over time thereof, biomarkers are needed which can be provided by advanced MRI techniques. This review summarizes to what extent MRI, especially advanced multiparametric techniques, can elucidate the underlying neuronal substrate that reflects the cognitive decline in type 2 diabetes.

Keywords: type 2 diabetes mellitus, magnetic resonance imaging, cognition, functional MRI, multiparametric MRI

INTRODUCTION

Type 2 diabetes mellitus is a common metabolic disorder, characterized by chronic hyperglycemia, in a context of insulin resistance and relative insulin deficiency (Gispen and Biessels, 2000). Type 2 diabetes has commonly been considered a disease of elderly populations. However, with today's unhealthy lifestyle, also an increasing number of younger (that is, middle-age) people are developing diabetes.

Type 2 diabetes has a broad range of serious clinical complications, including nephropathy, retinopathy, and cardiovascular disease, and is often accompanied by cardiovascular risk factors such as hypertension and dyslipidemia. Hyperglycemia damages a selection of cell types, including neurons, which are unable to reduce the transport of glucose inside the cell, leading to high glucose (Brownlee, 2005). Type 2 diabetes is also associated with cognitive deficits, accelerated cognitive decline, an increased risk of dementia, and Alzheimer disease (AD) (Biessels et al., 2006). In type 2 diabetes, cognitive changes mainly affect learning, memory and information processing speed

(Cheng et al., 2012). For recent reviews on cognition and type 2 diabetes, the reader is referred to specific recent reviews by Koekkoek et al. (2014) and Geijselaers et al. (2015).

In recent years, numerous studies have highlighted the adverse effects of diabetes on brain physiology and cognitive function to assess contributing pathophysiological mechanisms (Biessels and Reijmer, 2014; Brundel et al., 2014a). Most studies have applied conventional MRI with multiple contrasts to detect macrostructural cerebral changes. However, macrostructural abnormalities on MRI reflect end-stage effects of impaired tissue, and conventional MRI is probably not sensitive enough to detect the earliest cerebral changes, expectedly more closely reflecting mechanisms, associated with cognitive decline (Tofts, 2003). For this purpose, potentially more-sensitive MRI techniques, such as functional MRI (fMRI) and diffusion MRI (dMRI), can be used, which could lead to a better insight into the mechanisms that precede macrostructural (end-stage) abnormalities.

The present narrative review summarizes recent literature and provides an overview of the various brain abnormalities associated with type 2 diabetes in combination with cognitive decrements. The aim is to provide the available evidence for neuronal substrates of cognitive impairment in type 2 diabetes. It will explore the appropriate MRI techniques to study associations with cognitive performance in patients with type 2 diabetes (for an overview of typical abnormalities and the corresponding techniques, see **Figure 1**), and will make recommendations for future research. This review is structured according to the various types of cerebral abnormalities and the appropriate MRI techniques available to study pathophysiology, in the range from routine clinical application to explorative research.

ATROPHY

Cerebral atrophy can generally be defined as the shrinkage of brain tissue, which is a result of neurodegenerative processes, such as the loss of neurons and their interconnections (Jobst et al., 1994). Many studies on type 2 diabetes, using various structural MRI techniques, report on atrophy (den Heijer et al., 2003; de Bresser et al., 2010; van Elderen et al., 2010). Associations have been found between brain atrophy and decreased performance in various cognitive domains (Tiehuis et al., 2009; Hayashi et al., 2011; Moran et al., 2013; Zhang Y. et al., 2014), including memory, attention and executive function, as well as processing speed, motor speed, and sensory speed. Also the progression of atrophy was found related to cognitive decrements in type 2 diabetes (van Elderen et al., 2010; Reijmer et al., 2011).

SMALL VESSEL DISEASE

Cerebral small vessel disease (cSVD) can be generally defined as pathological processes with various etiologies that affect the small arteries, arterioles, venules, and capillaries of the brain (Wardlaw et al., 2013). Signs of cSVD are white matter lesions, microbleeds, silent brain infarcts and lacunar abnormalities, which are also indicative for cognitive decline (Imamine et al., 2011).

White Matter Lesions

White matter lesions (WMLs) are typically observed as regions of bright, high-signal intensity in the white matter (i.e., white matter hyperintensities) depicted on T2-weighted and, especially, FLAIR images (Wardlaw et al., 2013). The underlying pathophysiology of WMLs is still poorly understood and is assumed to include multiple factors of vascular (through ischemia or arteriosclerosis) or inflammatory (through transudation of CSF) origin (Fazekas et al., 1998).

WMLs are often divided in periventricular WMLs, which are located close to the ventricles, and deep WMLs, which are located in subcortical gray matter (Wardlaw et al., 2013). It was shown that periventricular, but not subcortical, WMLs are associated with the rate of cognitive decline in elderly non-demented individuals (De Groot et al., 2002).

Numerous studies report on WMLs in patients with type 2 diabetes (Manschot et al., 2006; Jongen et al., 2007; van Harten et al., 2007; Imamine et al., 2011). More specific, deep (subcortical) WMLs, periventricular WMLs, and WMLs in general are found in patients with type 2 diabetes. WMLs are also related with impaired cognition in type 2 diabetes (Manschot et al., 2006; Jongen et al., 2007; van Harten et al., 2007; Imamine et al., 2011), especially in the domains of processing speed, memory, attention and executive functioning, and motor speed.

Microbleeds

Cerebral microbleeds result from focal leakages of small blood vessels (Wardlaw et al., 2013). They are thought to contain iron deposits (Wardlaw et al., 2013). Typically, microbleeds are found only incidentally on MRI, but are thought to play an important role in cognitive decline (Wardlaw et al., 2013). The reported prevalence of microbleeds increases with age (Imamine et al., 2011). Microbleeds do not seem to be associated with type 2 diabetic patients with cognitive impairment (Moran et al., 2013), which is also confirmed at high field (7T) (Brundel et al., 2014b).

Silent Brain Infarcts

Silent brain infarcts (SBIs) are clinically asymptomatic (i.e., they lack stroke-like symptoms), but visible (generally 2–5 mm in diameter) as focal lesions on MRI, and are associated with cognitive deficits that commonly remain unnoticed (vermeer et al., 2007).

Patients with type 2 diabetes often display SBIs, which are also related to impaired cognitive performance (Manschot et al., 2006; Imamine et al., 2011). The number of SBIs and/or progression of SBIs are especially linked to decrements in motor speed, attention and executive functioning (Imamine et al., 2011; Umegaki et al., 2011).

Lacunar Abnormalities

Lacunes are pathologically defined as small areas (3–15 mm in diameter) of infarction, which is a result from an occlusion of one of the small penetrating branches of large cerebral arteries (Wardlaw et al., 2013) and are associated with cognitive impairment (Schneider et al., 2003). In type 2 diabetes, lacunar infarcts often progress (van Harten et al., 2006; Umegaki, 2010), likely caused by ischemia (Imamine et al., 2011).

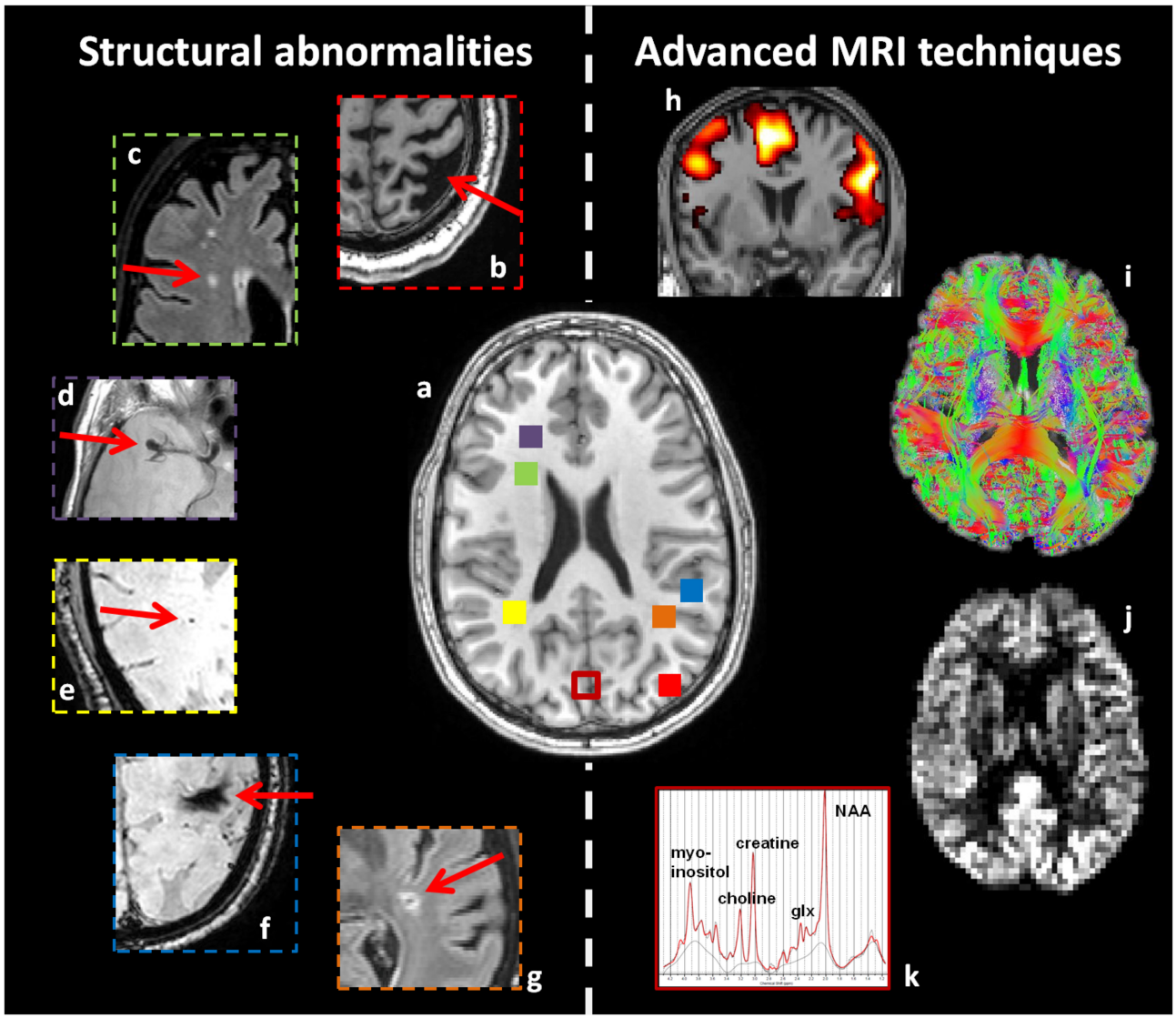

FIGURE 1 | Overview of structural abnormalities which may be found in patients with type 2 diabetes (b–g), and advanced MRI techniques sensitive to more subtle cerebral alterations **(h–k)**. This figure is an illustration from the authors' clinic. **(a)** T1-weighted image of a healthy young brain. Structural abnormalities in patients with type 2 diabetes, as highlighted with red arrows. **(b)** Atrophy (T1WI), **(c)** white matter lesions (FLAIR), **(d)** aneurysm (T2WI), **(e)** microbleeding (T2*-weighted), **(f)** macrobleeding (T2*-weighted), and **(g)** lacunar infarct (FLAIR). Advanced MRI techniques: **(h)** fMRI, **(i)** dMRI, **(j)** arterial spin labeling, and **(k)** MRS. Corresponding colored squares in **(a)** represent the approximate location where the structural abnormalities were found and where the single voxel for spectroscopy was located, respectively.

Cerebral infarcts (i.e., lacunar, cortical, subcortical infarcts, or infarcts in general) have been observed in patients with type 2 diabetes (Manschot et al., 2006; Moran et al., 2013). Most studies report a relationship between cerebral infarcts and decreased performance in various cognitive domains, including processing speed, sensory speed, memory, executive function, and global cognition.

For the detection of cerebral atrophy or cSVD, various structural MRI techniques have been used. However, these techniques cannot unravel more subtle details of tissue alterations that underlie or precede the atrophy or cSVD. For this, more-advanced MRI techniques can be used, which will be discussed below.

IMPAIRED CEREBRAL PERFUSION

Cerebral perfusion is defined as the amount of blood flowing through a definite volume of tissue in a given time (Filippi et al., 2010) and can be estimated using Arterial Spin Labeling (ASL) and Intravoxel Incoherent Motion (IVIM) imaging, or measured globally using a velocity-sensitive, phase-contrast MRI technique (Tiehuis et al., 2008; Brundel et al., 2012b; Novak et al., 2014; Rusinek et al., 2015; van Bussel et al., 2015; Jansen et al., 2016). The ASL technique is based on magnetic labeling of arterial blood (e.g., blood in the common carotid artery), which is used as an temporary endogenous tracer in the brain. The IVIM technique enables assessment of both the parenchyma and

microvasculature and is based on the diffusion of water molecules in parenchyma and incoherent motion of water molecules in the microvasculature. The velocity-sensitive, phase-contrast MRI technique is based on differences in phase of the magnetic spins. An advantage of using ASL or IVIM is that these techniques make it possible to investigate regional differences related to disease pathology instead of only a gross measurement of total brain perfusion with phase-contrast MRI (Le Bihan et al., 1986; Ryan et al., 2014).

A fair comparison between ASL and IVIM is not trivial due to the different complex physical mechanisms that contribute to the detected signal. However, the former is a truly quantitative method, which has been validated with PET (positron emission tomography) perfusion measurements (van Golen et al., 2014), whereas the latter is yet more experimental, though it can provide a higher signal-to-noise ratio (SNR), and the possibility for an increased spatial resolution.

Thus far, some studies, one using phase contrast MRI (1.5T) (Tiehuis et al., 2008) and one using ASL (3T) (Rusinek et al., 2015), did not find any differences in global perfusion between patients with type 2 diabetes and controls, while other studies (ASL, Novak et al., 2014; Xia et al., 2015a and IVIM, van Bussel et al., 2015, all at 3T) observed regional differences in perfusion. Possibly, differences in MRI methodology could explain these conflicting results. Atrophy can be a big confounder when assessing hypoperfusion using ASL, indeed most results disappear after correction for atrophy (Jansen et al., 2016). A recent ASL study applied a new analysis approach tallying the "distributed deviating voxels," and hypoperfusion was found in patients with type 2 diabetes, which remained significant after correction for atrophy in the subcortical gray matter (Jansen et al., 2016).

One phase contrast MRI study observed a positive association between perfusion and cognition, but this study was not able to explain the link of diabetes with cognitive performance (Tiehuis et al., 2008). Some studies did find a relationship between perfusion and impaired cognition in patients with type 2 diabetes (Brundel et al., 2012b; Xia et al., 2015a), although another study did not find this relationship (Jansen et al., 2016). Promising results regarding reduced cerebral perfusion in the insula cortex and cognitive performance were shown in a pilot ASL study (Novak et al., 2014). After insulin administration, memory and verbal fluency improved, and perfusion was elevated in the insula cortex of participants with diabetes, suggesting the involvement of an insulin mechanism. In type 2 diabetes, perfusion of the global gray matter was positively associated with verbal fluency (Rusinek et al., 2015), although local hippocampal perfusion (as measured using IVIM) had a negative association with memory performance (van Bussel et al., 2015). These results suggest the involvement of a vascular mechanism, and that the association might be dependent on the brain region.

Taken together, all perfusion techniques observed a relation with cognitive performance, which highlights the link between a vascular mechanism and cognitive decline. However, to observe regional differences in perfusion, the more-advanced MRI techniques (i.e., ASL and IVIM) appear more sensitive to contribute to the understanding of cognitive decline in patients with type 2 diabetes.

NEURONAL DYSFUNCTION

Neuronal dysfunction refers to all impairments of the neuronal system, including reduced functional activity of certain brain regions and connectivity between different regions (Zhou et al., 2010). Functional MRI (fMRI) offers the opportunity to investigate to which extent neuronal regions are active, in terms of blood oxygenation changes. The underlying principle is that neuronal activity leads to locally increased blood flow and oxygenation. Previous studies using the amplitude of low frequency fluctuations (ALFF), a measure of spontaneous neuronal activity, regional homogeneity, a measure of the neural regional synchronization, and functional connectivity, assessed by correlating time signals from distinct brain regions, reported on abnormal brain activity in patients with type 2 diabetes (Zhou et al., 2010; Musen et al., 2012; Xia et al., 2013; Cui et al., 2014).

Functional Connectivity

Reduced functional connectivity in the default mode network (DMN), i.e., the network of active brain regions when the brain is at rest and the participant is not focusing on anything particular, has been observed in patients with type 2 diabetes (Zhou et al., 2010; Musen et al., 2012; Chen et al., 2014, 2015, 2016; Hoogenboom et al., 2014; Cui et al., 2015; Xia et al., 2015b; Zhang H. et al., 2015). Moreover, reduced functional connectivity between the hippocampus and widespread regions in the DMN (Zhou et al., 2010), including the medial frontal cortex (Zhang H. et al., 2015) has been reported, in addition to reduced functional connectivity between the posterior cingulate and the medial frontal gyri and other regions in the DMN (Musen et al., 2012; Hoogenboom et al., 2014). Furthermore, reduced connectivity within the attention networks has been described (Xia et al., 2015b), which was associated with neuropsychological scores and glycated hemoglobin. Reduced connectivity of the DMN was related to impaired memory (Zhou et al., 2010; Zhang H. et al., 2015), executive function (Zhou et al., 2010), verbal fluency (Zhang H. et al., 2015), and lower global cognition (Zhang H. et al., 2015). The disrupted functional connectivity in the DMN has been shown to be inversely correlated with insulin resistance (Musen et al., 2012) in type 2 diabetes, hinting at an underlying insulin-related mechanism. This thought is enhanced by the observation of acutely increased functional connectivity between the hippocampus and multiple regions in the DMN after intranasal insulin administration (Zhang H. et al., 2015).

Interestingly, it was recently shown that participants with type 2 diabetes displayed altered fMRI network measures, characterized by a higher efficiency, compared with control participants (van Bussel et al., 2016c). Also subjects with pre-diabetes were studied, whose network measures fell between those with diabetes and control participants. The authors suggested that functional reorganization of the cerebral networks might act as a compensatory mechanism for cognitive decrements (van Bussel et al., 2016c).

Signal Fluctuations

ALFF and regional homogeneity alterations have been reported in a variety of DMN brain regions (including temporal lobe and frontal lobes) in patients with type 2 diabetes (Xia et al., 2013; Cui et al., 2014; Zhou et al., 2014). The altered ALFF and regional homogeneity values were related to impaired cognition, especially in the domains of attention and executive function (Xia et al., 2013; Cui et al., 2014; Zhou et al., 2014), speed (Xia et al., 2013; Cui et al., 2014), memory (Cui et al., 2014), and global cognition (Zhou et al., 2014). Moreover, ALFF values in the middle temporal gyrus were also inversely related to glycated hemoglobin (Xia et al., 2013) and insulin resistance in the diabetic group was negatively correlated with altered neuronal activity (Cui et al., 2014).

Brain Activation

Altered brain activation has also been found in patients with type 2 diabetes during a memory task, especially in task-related regions of the DMN (Marder et al., 2014), frontal cortex (Chen et al., 2014; Marder et al., 2014; He et al., 2015), parietal cortex (He et al., 2015) and the fronto-parietal network (Zhang Y. et al., 2016). Moreover, functional activation or connectivity is not only associated with memory performance (Zhang Y. et al., 2016), but also insulin resistance (Marder et al., 2014; Xia et al., 2015c), glycated hemoglobin (Marder et al., 2014; He et al., 2015), plasma glucose (Marder et al., 2014), and cholesterol (Xia et al., 2015d), suggesting a major role of glucose and lipid metabolism.

Overall, all functional MRI studies consistently show evidence of altered neuronal activity or functional connectivity in patients with type 2 diabetes and cognitive decrements.

WHITE MATTER TRACT ABNORMALITIES

White matter tract abnormalities refer to impaired integrity or altered organization of axonal bundles and can be investigated using diffusion MRI (dMRI). This technique is based on diffusion of water molecules, and during the dMRI acquisition, tissue is sensitized with the local characteristics of molecular diffusion. The measures most often analyzed by dMRI are fractional anisotropy (FA) and apparent diffusion coefficient (ADC). FA is a measure of tract directionality and ADC is a measure of water diffusivity. Clinically, an increase in ADC has been associated with reduced (neuronal) cell packing and increased extracellular space, possibly due to failure of neurogenesis or cell loss (Eriksson et al., 2001). Recently, analysis methods have become available that allow the assessment of the integrity and efficiency of structural networks, using graph theoretical analysis on dMRI data (Reijmer et al., 2013b).

Local Alterations

Microstructural abnormalities have been published for various brain regions in type 2 diabetes (Yau et al., 2009, 2010; Falvey et al., 2013; Zhang J. et al., 2014, 2016; van Bussel et al., 2016b; Xiong et al., 2016). Reduced FA has been observed in the white matter (Yau et al., 2010; Falvey et al., 2013) mostly concentrated in frontal and temporal regions (Yau et al., 2009), while elevated ADC values were found in a number of brain regions, including the hippocampus (Falvey et al., 2013) and multiple gray matter regions (Yau et al., 2010). Temporal lobe abnormalities were associated with impaired memory (Yau et al., 2009; van Bussel et al., 2015).

Network Alterations

Altered network and structural connectivity in type 2 diabetes have been shown using tractography (Reijmer et al., 2013a,b; Hoogenboom et al., 2014; van Bussel et al., 2016b; Yang et al., 2016; Zhang J. et al., 2016). Local and global network properties (i.e., cluster coefficient, global efficiency, path length) were altered and associated with impaired processing speed (Reijmer et al., 2013b). Elevated ADC and reduced FA were found in different tracts, including the superior longitudinal fasciculus (Reijmer et al., 2013a), uncinate fasciculus (Reijmer et al., 2013a; Hoogenboom et al., 2014), inferior longitudinal fasciculus (Reijmer et al., 2013a), corpus callosum (Reijmer et al., 2013a), and cingulum bundle (Hoogenboom et al., 2014). These tract abnormalities were associated with impaired processing speed and memory (Reijmer et al., 2013a; Hoogenboom et al., 2014) and highlight an underlying glucose-mediated mechanism as glycated hemoglobin and fasting blood glucose were also related to these tract abnormalities (Hoogenboom et al., 2014). Moreover, altered hippocampal white matter connectivity appear to be associated with memory decrements and type 2 diabetes (van Bussel et al., 2016b).

Diffusion MRI studies implicate that patients with type 2 diabetes show evidence of white matter microstructure, tract, and network abnormalities.

METABOLIC DYSFUNCTION

Proton magnetic resonance spectroscopy (^{1}H-MRS) enables the assessment of metabolic changes through the identification and quantification of spectral peaks associated with tissue metabolites (Jansen et al., 2006). ^{1}H-MRS is often used to investigate N-acetylaspartate (NAA), Choline (Cho), Creatine (Cr), myo-inositol (mIns), γ-aminobutyric acid (GABA), and glutamate. NAA is a measure of neuronal integrity and a surrogate marker of normal functioning neurons. Cho is an indirect marker of myelination and cell membrane metabolism. Cr is a measure of energy metabolism, and mIns has been proposed as a glial marker and as an end-product of persistent hyperglycaemia (Jansen et al., 2006). GABA and glutamate are major inhibitory and exhibitory neurotransmitters, respectively. However, *in vivo* detection and quantification of these neurotransmitter concentrations at low field strengths (<3T) are complicated due to spectral overlap with other metabolites. Another relevant metabolite in the context of diabetes is glucose, which typically requires high field strengths (>3T) for reliably detection with ^{1}H-MRS (Gruetter et al., 1996). An alternative method to study brain glucose levels using MR spectroscopy is ^{13}C-MR spectroscopy (van De Ven et al., 2012).

MR spectroscopy studies on type 2 diabetes in relationship with cognition have thus far been proven to be challenging, and often no associations between metabolic alterations and cognitive performance were found (Haroon et al., 2009; Tiehuis et al., 2010). However, a recent study found higher GABA+ levels

in participants with type 2 diabetes, and higher GABA+ levels in participants with both high HbA1c levels and less cognitive performance (van Bussel et al., 2016a). The authors concluded that participants with type 2 diabetes have alterations in the GABAergic neurotransmitter system, which are related to lower cognitive functioning, which hints at the involvement of an underlying metabolic mechanism.

INTERPRETATION

Table 1 provides an overview of all studies on type 2 diabetes, in which cognitive performance is related to diverse cerebral MRI contrasts. From this it can be appreciated that neuroradiologically visible MRI biomarkers (atrophy, WMLs, and lacunar abnormalities) and more subtle abnormalities (impaired cerebral perfusion, neuronal dysfunction, and white matter tract abnormalities) are related to cognitive decline, with a striking agreement between studies. For the other abnormalities (including microbleeds, SBIs, and metabolic dysfunction) the evidence of relationships with cognition is less convincing.

Most studies are associated with various methodological limitations. Most notably, often only a limited number of subjects is included. Furthermore, the studies show a pronounced diversity regarding subject selection, matching of subjects, diagnosis and classification of diabetes, adjustment for risk factors, and data analysis methods. Due to the different designs and limited number of available studies, it is difficult for studies reporting negative results to assess whether the applied techniques (or study methods) are not sensitive enough to pick up cognitive performance-related alterations, or whether these alterations are not present at all. Interestingly, in those studies where cerebral changes were found, these were most often located in the frontal and/or temporal lobe (den Heijer et al., 2003; Zhou et al., 2010, 2014; Musen et al., 2012; He et al., 2015; van Bussel

TABLE 1 | Overview of neuroimaging abnormalities associated with cognitive performance in type 2 diabetes mellitus.

Brain abnormalities	MRI techniques	Major outcomes	References
CLINICAL APPLICATIONS			
Atrophy	– T1WI – T2WI – FLAIR – IR	Cerebral atrophy increases with cognitive decline	den Heijer et al., 2003; Manschot et al., 2006; van Elderen et al., 2010; Hayashi et al., 2011; Reijmer et al., 2011
SMALL VESSEL DISEASE			
White matter lesions	– T2WI – FLAIR	White matter lesion load increases with cognitive decline	Manschot et al., 2006; Jongen et al., 2007; van Harten et al., 2007; Imamine et al., 2011
Microbleeds	– T2*WI	No evidence of microbleeds with cognitive decline	Moran et al., 2013; Brundel et al., 2014b
Silent brain infarcts	– T1WI – T2WI – FLAIR	Progression of silent brain infarcts seems related to cognitive decline	Imamine et al., 2011; Umegaki et al., 2011
Lacunar abnormalities	– T1WI – T2WI – FLAIR	Cerebral ischemic lesions are related to cognitive decline	Manschot et al., 2006; Umegaki, 2010
Impaired cerebral perfusion	– ASL – PC-MRI – IVIM	Diverse results regarding perfusion in diabetes. Perfusion related to cognitive decline	Tiehuis et al., 2008; Brundel et al., 2012b; Novak et al., 2014; Rusinek et al., 2015; Xia et al., 2015a; van Bussel et al., 2015; Jansen et al., 2016
NEURONAL DYSFUNCTION			
Functional connectivity	– fMRI (connectivity)	Reduced functional connectivity in relationship with cognition; higher efficiency in T2DM with cognitive decrements	Zhou et al., 2010; Xia et al., 2015b; Zhang Y.-W. et al., 2015; van Bussel et al., 2016c
Signal fluctuations	– ALFF	Altered ALFF related to impaired cognition	Xia et al., 2013; Cui et al., 2014; Zhou et al., 2014
Brain activation	– fMRI (activation)	Altered neuronal activity in relationship with cognitive decline	Zhang Y. et al., 2016
WHITE MATTER TRACT ABNORMALITIES			
Local alterations	– dMRI (diffusion measures)	Temporal lobe abnormalities were associated with impaired memory	Yau et al., 2009; van Bussel et al., 2016b
Network alterations	– dMRI (connectivity)	Tract abnormalities and network alterations related to impaired cognition	Reijmer et al., 2013a,b; Hoogenboom et al., 2014; van Bussel et al., 2016b
Metabolic dysfunction	– MRS	Insufficient evidence regarding metabolic alterations and cognitive performance	Haroon et al., 2009; Tiehuis et al., 2010; van Bussel et al., 2016a

Only MRI references in combination with cognitive performance are included in this table. T2(*)WI, T2(star)-weighted images; IR, inversion recovery images; ASL, arterial spin labeling; PC-MRI, (velocity-sensitive) phase-contrast MRI; IVIM, intravoxel incoherent imaging; fMRI, functional MRI; ALFF, amplitude of low frequency fluctuations; dMRI, diffusion MRI; MRS, magnetic resonance spectroscopy.

et al., 2015), which is in close agreement with the type of cognitive decline typically experienced in type 2 diabetes (Gold et al., 2007).

Type 2 diabetes is also known to increase the risk of developing AD (Steen et al., 2005; Cheng et al., 2012). MRI studies show that gray matter loss, insulin resistance, and medial temporal lobe atrophy are associated with AD (Thompson et al., 2003; Biessels et al., 2006), traits also present in patients with type 2 diabetes (den Heijer et al., 2003). These results suggest that diabetes might to some extent be linked to Alzheimer's Disease (AD) and that diabetes and AD might share similar mechanisms underlying cognitive decline (Ryan et al., 2014).

FUTURE OUTLOOK

As the neuronal mechanisms underlying cognitive decline associated with type 2 diabetes still remain to be elucidated, and studies using more-advanced and potentially more-sensitive MRI techniques are scarce, intensified research is needed to investigate the underlying mechanisms of brain damage (Jouvent et al., 2010). It will also be interesting to investigate cognitive decline in pre-diabetic stages such as the metabolic syndrome or impaired glucose mechanism (Grundy, 2006; van Bussel et al., 2016c).

In addition to the imaging techniques discussed in this review, other novel MRI approaches might also yield interesting new biomarkers, such as Dynamic Contrast Enhanced MR Imaging, which is an MRI technique where T1-weighted scans are acquired dynamically after injection of a contrast agent, and pharmacokinetic modeling of the enhancing tissue signal can provide information about physiological tissue characteristics, including BBB integrity in terms of leakage of contrast medium (Taheri et al., 2011). It could be relevant to study the role of BBB in diabetes, because disruption of the BBB is also considered to be a result of cSVD.

Furthermore, metabolites that are relatively difficult to detect, such as GABA, dedicated MRS spectral editing sequences exist to identify and quantify these metabolite concentrations (Puts and Edden, 2012). The use of a specifically designed MRS acquisition scheme allows for the selective recording of signals only from the desired metabolite, while other metabolites are eliminated.

Another important direction is the application of high field MRI (Brundel et al., 2012a), as most studies in this review were performed at 1.5T. High field MRI ($\geq$3T) has several benefits as it provides higher spatial resolution and improved SNR, although it is more susceptible for artifacts. Additionally, future studies should incorporate a multiparametric approach, to provide a more complete picture of the locations and nature of affected cerebral regions. Also, analysis approaches for fMRI and dMRI should focus on cerebral networks, as cognitive functions affected by diabetes correspond to networks, rather than localized brain regions.

Additionally, future, preferably large multicenter studies, are required to validate current findings, or provide a more definitive answer regarding issues for which currently contradictory findings have been reported in different studies (such as the inconsistencies reported regarding type 2 diabetes and global perfusion). For this, quantitative measures are essential, regarding both quantitative MRI as neuropsychological tests to characterize and define in more detail the cognitive status of the population under investigation.

CLINICAL RELEVANCE

The application of neuroimaging techniques to study diabetes associated accelerated cognitive decline is relevant as we expect to obtain new insights regarding affected brain regions, networks, and tissue abnormalities. Furthermore, MRI measures might provide early biomarkers for cognitive decline (see **Table 1** for an overview), and could potentially be used to identify patients at risk. Follow-up studies can be performed to confirm that subjects with sufficient cerebral MRI alterations eventually develop cognitive problems, and one could consider an interventional study with a combination of diet, exercise or medication (Zhang H. et al., 2015) to explore whether cerebral MRI alterations also delay, or even improve, after intervention (Raji et al., 2015). Hence, by performing advanced neuroimaging, a more complete picture can be obtained of the effect of diabetes on the brain, it might provide a better timing of (preventive) therapy, and it could shed some light on the course and efficacy of the therapy to prevent or halt cognitive decline.

CONCLUSIONS

Cognitive decline in type 2 diabetes is associated with brain alterations, which can be detected using neuroimaging. The battery of MRI techniques available to study this topic is highly versatile, and several aspects of brain function and integrity can be studied noninvasively. Advanced, novel MRI techniques are expected to reveal more subtle brain alterations compared with only structural MRI. Therefore, more-advanced multiparametric MRI techniques should be implemented in future studies to investigate the role of diabetes on cognitive performance, and the underlying pathophysiological mechanisms.

LITERATURE SEARCH

We searched PubMed for articles published until September 19, 2016, with the following terms and combinations of these terms: "arterial disease," "arterial spin labeling," "atrophy," "axon damage," "brain," "cerebral," "cogniti*," "connectivity," "diabet*," "diffusion tensor imaging," "DTI," "fMRI," "functional MRI," "imaging," "lacun*," "lacunar infarct," "microbleeds," "microstructural abnormalit*," "MRI," "MRS," "MR spectroscopy," "neuronal dysfunction," "neuronal function," "neuropathy," "perfusion," "syndrome," "type 2," "vessel disease," "white matter lesion."

We included articles identified from these searches and relevant references cited in the articles.

The neuropsychological terminology is subdivided in (1) (verbal) memory, (2a) (information) processing speed, (2b) sensory speed, (2c) motor speed, (3) IQ, (4) global cognition, (5) attention functions, (6) executive functions, (7) psychomotor functions, (8) visuoconstruction, and (9) fluency, according to Hebben and Milberg (2009). Speed is subdivided into three

components: (1) processing speed (central part/brain), (2) sensory speed (visual aspects) and (3) motor speed (conducting part of a test/trail).

Animal studies, studies on patients with type 1 diabetes mellitus, and studies in which MRI results were presented without addressing correlations with cognitive performance were not included. Only articles written in English were included.

AUTHOR CONTRIBUTIONS

FCGvB searched for published reports and wrote the first draft of the Review. WB and JJ helped to improve the first draft with addition of relevant reports, suggestions for structure of the Review, and the idea for a schematic table. PH, Rv, Mv, FRJV, HS, MS, CS, and JW read the Review critically and made suggestions for improvements.

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Increased Short-Term Beat-to-Beat QT Interval Variability in Patients with Impaired Glucose Tolerance

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Prediabetic states and diabetes are important risk factors for cardiovascular morbidity and mortality. Determination of short-term QT interval variability (STV_{QT}) is a non-invasive method for assessment of proarrhythmic risk. The aim of the study was to evaluate the STV_{QT} in patients with impaired glucose tolerance (IGT). 18 IGT patients [age: 63 ± 11 years, body mass index (BMI): 31 ± 6 kg/m^2, fasting glucose: 6.0 ± 0.4 mmol/l, 120 min postload glucose: 9.0 ± 1.0 mmol/l, hemoglobin A1c (HbA1c): 5.9 ± 0.4%; mean ± SD] and 18 healthy controls (age: 56 ± 9 years, BMI: 27 ± 5 kg/m^2, fasting glucose: 5.2 ± 0.4 mmol/l, 120 min postload glucose: 5.5 ± 1.3 mmol/l, HbA1c: 5.4 ± 0.3%) were enrolled into the study. ECGs were recorded, processed, and analyzed off-line. The RR and QT intervals were expressed as the average of 30 consecutive beats, the temporal instability of beat-to-beat repolarization was characterized by calculating STV_{QT} as follows: $STV_{QT} = \Sigma|QT_{n+1} - QT_n|\ (30x\sqrt{2})^{-1}$. Autonomic function was assessed by means of standard cardiovascular reflex tests. There were no differences between IGT and control groups in QT (411 ± 43 vs 402 ± 39 ms) and QTc (431 ± 25 vs 424 ± 19 ms) intervals or QT dispersion (44 ± 13 vs 42 ± 17 ms). However, STV_{QT} was significantly higher in IGT patients (5.0 ± 0.7 vs 3.7 ± 0.7, $P < 0.0001$). The elevated temporal STV_{QT} in patients with IGT may be an early indicator of increased instability of cardiac repolarization during prediabetic conditions.

Keywords: cardiovascular autonomic neuropathy, impaired glucose tolerance, prediabetes, proarrhythmic risk, short-term variability of the QT interval, sudden cardiac death, QT dispersion, QT prolongation

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INTRODUCTION

Prediabetic states and diabetes are important risk factors for cardiovascular morbidity and mortality (1–3). Cardiovascular death or death of unknown origin was in the 0.4–0.5% range in the subgroups of a 3-year follow-up study on patients with impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG) level (4). In a 23-year follow-up study on Japanese American men, the relative risks for sudden cardiac death were 2.22 in subjects with asymptomatic hyperglycemia, and 2.76 in diabetic patients (5). Diabetes status was a strong risk factor for sudden death, but not for fatal

myocardial infarction in men during the population-based Paris Prospective Study I (6). Higher risk of sudden cardiac death was associated with borderline diabetes, diabetes with or without microvascular disease, compared to subjects without diabetes in a population-based case–control study of patients experienced out-of-hospital cardiac arrest due to heart disease (1). Out-of-hospital sudden cardiac deaths were 1.79-fold for non-diabetic men with impaired fasting plasma glucose and 2.26-fold for men with type 2 diabetes, and a 1 mmol/l increment in fasting plasma glucose was related to an increase of 10% in the risk of sudden cardiac death in Finnish men (7).

The prevalence of confirmed cardiovascular autonomic neuropathy (AN), an impairment of autonomic control of the cardiovascular system during diabetes, was between 16 and 20% in unselected type 1 and type 2 diabetic patients (8). Cardiovascular AN is a risk marker of cardiovascular morbidity, and it causes a 3.65-fold increase in the relative risk of mortality (8). Cardiac AN promotes ventricular repolarization disturbances [QTc prolongation, increased QT dispersion (QTd)] and may increase the risk of sudden cardiac death. Prolongation of QT interval could lead to increased myocardial electrical instability, predisposing diabetic subjects with AN to potentially fatal ventricular arrhythmias (9). Cardiac AN with QT interval prolongation proved to be a poor prognostic factor for sudden cardiac death in diabetic patients in a 5-year follow-up study (10). Prolonged QTc is more frequent in patients with IFG (30%) and with diabetes (42%) than in subjects with normal glucose tolerance (22%), and both IFG and diabetes increased the risk of prolonged QTc (11). QTc interval duration was found to be significantly higher both during the day and night using ECG Holter recordings in patients with IGT compared to subjects with normal glucose tolerance (12). IGT was confirmed in 15% of men and 23% of women with QTc prolongation (>440 ms) in the population-based Hisayama study in Japan (13).

In the clinical setting, the risk assessment of serious ventricular arrhythmias in individual patients is challenging since the prolongation of repolarization that manifests as QT interval prolongation on the ECG does not always correlate with subsequent development of ventricular arrhythmias (14–16). Cardiac repolarization reserve may be reduced even without significant changes in the duration of cardiac repolarization; therefore, QT interval prolongation cannot reliably predict the development of ventricular arrhythmias (17). The short-term variability of the QT interval (STV_{QT}) was introduced as an early and sensitive indicator of repolarization instability (18) that more reliably predicted ventricular arrhythmias and sudden cardiac death than prolongation of repolarization in previous experimental (16, 19–22) and clinical studies (23–27). Type 1 diabetes mellitus moderately lengthened ventricular repolarization, attenuated repolarization reserve, and enhanced the risk of sudden cardiac death in dogs (27, 28), and similar mechanisms might also occur in patients suffering from prediabetic states and diabetes.

The aim of the present study was to determine beat-to-beat STV_{QT} for assessment of repolarization instability and possible proarrhythmic risk, together with cardiovascular autonomic function in patients with IGT.

MATERIALS AND METHODS

Patient Population

Patients with IGT who are followed at the First Department of Medicine, Semmelweis University, Budapest, Hungary, were eligible for this study. Patients were excluded if they had excessive (>5%) ectopic atrial or ventricular beats, were in a rhythm other than normal sinus, had repolarization abnormalities (i.e., early repolarization pattern, T wave inversion, and complete left bundle branch block or right bundle branch block), had a permanent pacemaker or any other disorders such as serious retinopathy, symptomatic cardiac and pulmonary disease, and acute metabolic disease, had excessive noise on the electrocardiographic signal that precluded analysis of the ECG waveform, were on any medication likely to affect the investigated ECG parameters, or consumed significant amount of food within 3 h or drank alcohol, coffee, or smoked within 10 h.

We studied 18 IGT patients, 9 males and 9 females with the age of 63 ± 11 years (all values presented are mean $\pm$ SD). A total of 18 age- and sex-matched volunteers (mean age 56 ± 9 years) without a history or evidence of heart disease were enrolled in the study as controls. All of the control individuals and IGT patients were of Caucasian origin.

The studies described here were carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association and were approved by the Scientific and Research Ethical Committee of the Medical Research Council at the Hungarian Ministry of Health (ETT-TUKEB), under ethical approval No. 4987-0/2010-1018EKU (338/PI/010). All subjects have given written informed consent of the study.

Data Collection and Analysis

Before the ECG recording, all IGT patients and controls were at rest, in the supine position for 10 min. Then, 12-lead electrocardiograms were continuously recorded for 5 min at rest, also in the supine position to obtain signals with the least amount of motion artifact. In all leads, the ECG signals were digitized at 2,000 Hz sampling rate with a multichannel data acquisition system (Cardiosys-A01 software, MDE Heidelberg GMBH, Heidelberg, Germany) connected to a personal computer and stored for later off-line analysis.

Out of the repolarization parameters, we analyzed the frequency corrected QT interval (QTc) using Bazett's ($QTc = QT/\sqrt{RR}$), Fridericia ($QTc = QT/[RR/1{,}000]1/3$), Framingham ($QTc = QT + [0.154 \times \{1{,}000 - RR\}]$) and the Hodges formulas ($QTc = QT + 1.75 \times [60{,}000/RR - 60]$), the QTd, the PQ and QRS intervals, the duration of terminal part of T waves ($T_{peak} - T_{end}$) and the short-term variability of QT interval (STV_{QT}).

The RR and QT intervals, as well as duration of the T wave from the peak to the end ($T_{peak} - T_{end}$) intervals were measured semi-automatically in 30 consecutive beats (minimum number of intervals needed for variability measurements) and were calculated as the average of 30 beats. The QT intervals were analyzed by conventional computerized QT measurement technique, all QT intervals were checked in a blinded manner by the same expert investigator of the team and fiducial cursor positions set by the

software were manually corrected if needed (29). QTc interval duration was defined as the mean duration of all QTc intervals measured. The PQ and QRS intervals were measured as the average of 15 consecutive beats. All measurements were carried out using limb lead II and in case of excessive noise in limb lead II, lead V5.

To characterize the temporal instability of beat-to-beat heart rate (HR) and repolarization, Poincaré plots of the QT and RR intervals were constructed, where each QT and RR value is plotted against its former value. STV_{QT} and STV_{RR} were calculated using the following formula: $STV = \Sigma|D_{n+1} - D_n| \ (30x\sqrt{2})^{-1}$, where *D* represents the duration of the QT and RR intervals. This calculation defines the STV as the mean distance of points perpendicular to the line of identity in the Poincaré plot and relies on previous mathematical analysis (30).

Autonomic function was assessed by means of five standard cardiovascular reflex tests: the HR responses to deep breathing and to standing up (30/15 ratio), the Valsalva maneuver, the systolic blood pressure response to standing up, and the diastolic pressure change during a sustained handgrip (31). A score was created to express the severity of AN, based on the results of the five tests (normal: 0, borderline: 1, abnormal: 2). The total score was in the interval of 0–10.

Fasting venous blood samples were obtained from each patient and controls for the determination of serum glucose and hemoglobin A1c (HbA1c) levels. Oral glucose tolerance test (OGTT) was carried out with 75 g glucose to confirm the diagnosis of IGT according to the World Health Organization recommendation (120 min value in 7.8–11.0 mmol/l range).

Statistical Analysis

All data are expressed as mean ± SD. Comparisons between IGT patients and controls for the study variables were done using the unpaired Student's *t*-test for normally distributed parameters (D'Agostino-Pearson test was used to assess normality of distribution), and linear regression for revealing correlations. The statistical analyses were performed using the Statistica 12 software package. Statistical significance was defined by $P < 0.05$ level.

RESULTS

Clinical Data of IGT Patients and Control Subjects

In 18 IGT patients studied, mean body mass index (BMI) was significantly higher ($P < 0.05$) than among age- and sex-matched healthy volunteers. Mean systolic blood pressure did not differ significantly between control subjects and IGT patients; however, IGT patients had lower diastolic blood pressure (74 ± 9 vs 81 ± 10 mmHg; $P < 0.05$). Significant differences were seen between IGT and control groups in mean serum glucose (6.0 ± 0.4 vs 5.2 ± 0.4 mmol/l; $P < 0.0001$), HbA1c (5.9 ± 0.4 vs 5.4 ± 0.3%; $P < 0.0001$), and serum glucose 120 min level during OGTT (9.0 ± 1.0 vs 5.5 ± 1.3 mmol/l; $P < 0.0001$). Clinical data of IGT patients and control subjects are shown in **Table 1**.

TABLE 1 | Clinical data of IGT patients and age-matched control subjects.

	Control	Patients with IGT
n	18	18
Sex (male/female)	9/9	9/9
Age (year)	56 ± 9	63 ± 11
Weight (kg)	79 ± 19	88 ± 17
Height (cm)	170 ± 11	168 ± 6
BMI (kg/m^2)	27 ± 5	31 ± 6*
Systolic BP (mmHg)	130 ± 12	134 ± 17
Diastolic BP (mmHg)	81 ± 10	74 ± 9*
0 min glucose (mmol/l)	5.2 ± 0.4	6.0 ± 0.4**
120 min glucose (mmol/l)	5.5 ± 1.3	9.0 ± 1.0**
HbA1c (%)	5.4 ± 0.3	5.9 ± 0.4**

Values are represented as mean ± SD. Values are considered statistically significantly different at P < 0.05 (*), P < 0.0001 (**) compared with the control group.
IGT, impaired glucose tolerance; BMI, body mass index; BP, blood pressure; HbA1c, hemoglobin A1c.

TABLE 2 | Electrocardiographic parameters in patients with IGT and age-matched controls.

	Control	Patients with IGT
RR (ms)	900 ± 144	914 ± 163
PQ (ms)	161 ± 18	162 ± 24
QRS (ms)	94 ± 9	94 ± 8
QT (ms)	402 ± 39	411 ± 43
QTc (ms) Bazett	424 ± 19	431 ± 25
QTc (ms) Fridericia	416 ± 23	424 ± 27
QTc (ms) Framingham	417 ± 22	424 ± 26
QTc (ms) Hodges	416 ± 25	424 ± 29
QTd (ms)	42 ± 17	44 ± 13
$T_{peak} - T_{end}$ (ms)	86 ± 14	88 ± 23
T wave amplitude (μV)	220 ± 119	225 ± 120
STV_{RR} (ms)	18.5 ± 14.3	10.5 ± 6.7*
STV_{QT} (ms)	3.7 ± 0.7	5.0 ± 0.7**

Values are represented as mean ± SD. Values are considered statistically significantly different at P < 0.05 (*), P < 0.0001 (**) compared with the control group. n = 18 in each group.
IGT, impaired glucose tolerance; QTc, frequency corrected QT interval (calculated by the Bazett's, Fridericia, Framingham and Hodges formulas); QTd, QT dispersion; $T_{peak} - T_{end}$, duration of the T wave from the peak to the end; STV_{RR}, beat-to-beat short-term temporal variability of the RR interval; STV_{QT}, beat-to-beat short-term temporal variability of the QT interval.

Electrocardiographic Parameters in Study Subjects

Comparison of the two groups (IGT patients vs controls) revealed no significant differences in HR, the PQ, QRS, QT and $T_{peak} - T_{end}$ intervals and the QTd. In order to reliably assess the duration of ventricular repolarization and to minimize the influence of changing HR on the QT interval, the frequency corrected QT interval (QTc) was calculated by the Bazett's, Fridericia, Framingham and Hodges formulas. QTc values calculated with all the four formulas showed no significant differences between IGT patients and controls. Electrocardiographic parameters in study subjects are presented in **Table 2**.

Short-term Beat-to-Beat Variability of the QT and RR Intervals

As it has been shown that T wave amplitude may affect STV_{QT} (32), we have also compared the T wave amplitudes in both

groups. T wave amplitudes did not differ significantly between IGT patients and control subjects (225 ± 120 vs 220 ± 119 μV, $P = 0.882$).

To characterize the instability of cardiac ventricular repolarization, the short-term beat-to-beat variability of the QT interval was calculated in IGT patients and age-matched controls. Since it is reasonable to assume that STV_{QT} can be, at least in part, influenced by the short-term variability of the RR interval, the STV_{RR} was also calculated in both groups (33). Patients with IGT exhibited a significantly lower STV_{RR} compared to controls (10.5 ± 6.7 vs 18.5 ± 14.3 ms, $P = 0.0373$). No significant correlation was found between STV_{QT} and STV_{RR} values in IGT patients ($r = -0.3152$; $P = 0.203$).

As individual representative examples (Poincaré plots) illustrate (**Figure 1**) and grouped average data show (**Table 2**), STV_{QT} was significantly increased by 36% in IGT patients compared to controls (5.0 ± 0.7 ms vs 3.7 ± 0.7 ms, $P < 0.0001$).

Cardiovascular Autonomic Function

Standard cardiovascular reflex tests indicated significant deteriorations in Valsalva ratio ($P < 0.0001$) and the HR responses to deep breathing among IGT subjects compared to controls ($P = 0.033$). However, no significant differences in 30/15 ratio, systolic blood pressure response after standing up, diastolic blood pressure response after sustained handgrip, and AN score were detected between the two groups. Autonomic parameters of IGT patients and age-matched control subjects are shown in **Table 3**.

Correlation of Short-term QT Interval Variability (STV_{QT}) with Laboratory Data and AN Parameters in Patients with IGT

Pearson correlation coefficient values indicated that neither laboratory data nor autonomic parameters correlated with STV_{QT}, these data are presented in **Table 4**. However, 30/15 ratio had significant negative correlation with STV_{QT} ($r = -0.4729$; $P = 0.048$).

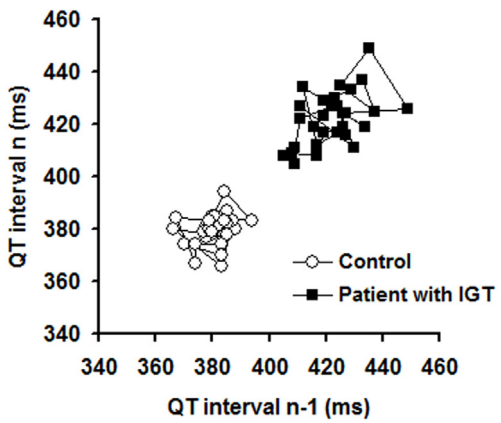

FIGURE 1 | Representative Poincaré plots illustrating short-term temporal variability of the QT interval in a control individual and in a patient with impaired glucose tolerance (IGT).

TABLE 3 | AN parameters of IGT patients and age-matched control subjects.

	Control	Patients with IGT
Heart rate (HR) variation during deep breathing (1/min)	16 ± 7	11 ± 8*
Valsalva ratio	1.7 ± 0.3	1.2 ± 0.1**
30/15 ratio	1.3 ± 0.3	1.2 ± 0.1
Systolic BP fall after standing up (mmHg)	8 ± 8	6 ± 7
Diastolic BP increase after sustained handgrip (mmHg)	11 ± 6	14 ± 6
AN score	2.4 ± 1.2	2.7 ± 1.3

Values are represented as mean ± SD. Values are considered statistically significantly different at P < 0.05 (*), P < 0.0001 (**) compared with the control group. n = 18 in each group.
IGT, impaired glucose tolerance; 30/15 ratio, immediate HR response to standing; BP, blood pressure; AN, autonomic neuropathy.

TABLE 4 | Correlation of short-term QT interval variability (STV_{QT}) with laboratory data and AN parameters in patients with IGT.

	STV_{QT} in patients with IGT (ms)	
	Pearson *r*	*P* value (two-tailed)
HbA1c (%)	0.2708	0.277
OGTT 0 min (mmol/l)	0.2118	0.399
OGTT 120 min (mmol/l)	−0.1118	0.659
Heart rate (HR) variation during deep breathing (1/min)	−0.0379	0.881
Valsalva ratio	0.1101	0.664
30/15 ratio	−0.4729	0.048*
Systolic BP fall after standing up (mmHg)	−0.0163	0.949
Diastolic BP increase after sustained handgrip (mmHg)	−0.0685	0.787
AN score	−0.1353	0.593

Values are represented as Pearson correlation coefficient. Values are considered statistically significantly different at P < 0.05 (*).
STV_{QT}, beat-to-beat short-term temporal variability of the QT interval; IGT, impaired glucose tolerance; HbA1c, hemoglobin A1c; OGTT, oral glucose tolerance test; 30/15 ratio, immediate HR response to standing; BP, blood pressure; AN, autonomic neuropathy.

DISCUSSION

Cardiac autonomic dysfunction present in prediabetes may lead to repolarization disturbances and may increase the risk of ventricular arrhythmias and sudden cardiac death. In this study, we show for the first time that beat-to-beat STV_{QT}, an early and sensitive parameter of repolarization instability, is increased even before QTc prolongation or enhanced QTd could be detected in patients with IGT.

Patients with prediabetic conditions or diabetes have higher risk for sudden cardiovascular death (1, 5–7). Cardiac AN and instability of cardiac repolarization, detected by QTc prolongation or increased QTd, contribute to the increased risk for sudden cardiac death (9, 10). Prolonged QTc was related to a progressive worsening of glucose tolerance after adjustment for possible confounding factors in elderly women with IGT or diabetes (34). Impairment of cardiac parasympathetic and sympathetic innervation as well as QT interval prolongation may play a partial role in the pathogenic mechanism of sudden unexpected death in diabetic patients. Cardiovascular adaptation mechanisms, including

baroreflex sensitivity and HR variability, are also impaired in diabetic AN that may further increase the risk for arrhythmia development (35).

However, decreased repolarization capacity and increased arrhythmia susceptibility is not necessarily preceded by significant changes in the duration of cardiac repolarization, and in these cases, cardiac repolarization reserve may be reduced without manifest QT interval prolongation (17). Importantly, a wide range of non-cardiovascular drugs or even dietary constituents with only mild repolarization blocking effects can increase the risk for serious ventricular arrhythmias and sudden cardiac death in patients with impaired repolarization reserve (17). Therefore, in this clinical setting, the prediction of lethal ventricular arrhythmias is especially challenging. STV_{QT} has been suggested as an early and sensitive indicator of temporal repolarization instability based on previous experimental and clinical studies (16, 18, 20, 24–26).

Our present study is the first to indicate that patients with IGT, a prediabetic condition, have repolarization instability indicated by elevated beat-to-beat STV_{QT}. This study was not designed to assess the exact mechanisms responsible for repolarization disturbances in patients with IGT; however, several possible mechanisms may be considered. Compelling recent evidence suggests a direct link between type 2 ryanodine receptor (RyR2) dysfunction in the endo/sarcoplasmic reticulum leading to altered intracellular calcium homeostasis, glucose intolerance, and impaired insulin secretion in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT) (36, 37). The known *RYR2* mutations identified in these CPVT patients were previously linked to reduced binding affinity of calstabin2 to the RyR2 channel resulting in intracellular Ca^{2+} leak (37–39). In knock-in mouse models where these CPVT-linked mutations leading to RyR2-mediated Ca^{2+} leak were reconstituted, mitochondrial dysfunction and blunted ATP production with concomitantly increased sarcolemmal K_{ATP} channel function (reversible by the K_{ATP} blocker glibenclamide) were found in pancreatic β-cells to cause reduced insulin secretion and consequently, IGT (36). In addition to causing altered glucose metabolism and providing triggers for cardiac arrhythmias (CPVT), the RyR2-mediated Ca^{2+} leak—by depleting Ca^{2+} stores—may also contribute to arrhythmia substrate creation *via* reduced I_{Ks} current, i.e., decreased Ca^{2+}-dependent I_{Ks} activation (40) and consequently, impaired repolarization reserve (17). Interestingly, and in accordance with this mechanism, reduced I_{Ks} density, impaired repolarization reserve, and increased risk for sudden cardiac death were described in diabetic dogs (28). Although there is no doubt that RyR2 channel dysfunction is directly linked to heart failure (41), cardiac arrhythmia development (42, 43), IGT, and reduced insulin release (36, 44), however, further clinical studies are needed to determine whether *RYR2* mutations leading to leaky RyR2 channels are frequently present in patients diagnosed with IGT in general.

Repolarization instability can be a long-standing risk factor for cardiovascular morbidity and mortality in prediabetic states and during development of diabetes. However, the role of additional cardiovascular risk factors cannot be excluded in early prediabetic conditions. Early sympathetic nerve dysfunction and insulin resistance may also play a role in the development of decreased coronary flow reserve in patients with normoglycemia (45). In this regard, increased QT interval variability associated with sympathetic dysinnervation was observed in patients with type 2 diabetes in the supine position and the QT variability was further elevated in the context of sympathetic activation upon standing (46).

Relative sympathetic predominance was observed in cardiovascular reflex tests during IGT, as sympathetic parameters (systolic BP fall after standing up and diastolic BP increase after sustained handgrip) were unchanged, whereas two of three parasympathetic parameters measured (HR variation and Valsalva ratio) were significantly decreased. In addition, a significant negative correlation was seen between the values measured in the third parasympathetic test (30/15 ratio) and STV_{QT} in our study. The significantly lower STV_{RR} values observed also represent this parasympathetic dysinnervation and subsequent relative sympathetic predominance in patients with IGT. Sympathetic predominance acutely evoked by graded head-up tilt test resulted in similar changes, such as decreased variance of HR and increased variance of repolarization duration (47, 48).

The prevalence of distal symmetric polyneuropathy that may result in weakness, sensory loss, pain, autonomic dysfunction, gait impairment, falls, and disability has been reported to be 11% in patients with IGT (49). It is known that IGT is present in about 40% of patients with idiopathic peripheral neuropathy and abnormal microvascular endothelial dysfunction is common in both patient groups (50). It has long been known that IGT is associated with AN and a shift is observed in sympathovagal balance to sympathetic overactivity (51–54). Prevalence of parasympathetic dysfunction was 25%, whereas the prevalence of sympathetic dysfunction was 6% in 268 patients with IGT in the Finnish Diabetes Prevention Study (55). Abnormal sinus arrhythmia test (55 vs 33%; $P = 0.004$) and abnormal Valsalva maneuver (34 vs 7%; $P = 0.004$) were significantly more frequent in patients with IGT than in control subjects; however, the frequency of abnormal postural test was not different in these two groups ($P = 0.334$) (51). Insulin resistance was associated with global autonomic dysfunction and an increased LF/HF (low frequency/high frequency) ratio indicating sympathetic overactivity (52). However, the autonomic dysfunction was less significant in IGT patients than in diabetic subjects (52). IGT induced decrease in parasympathetic modulation (decreased HF power and 30/15 ratio) and a shift toward augmented sympathetic tone (increased LF/HF ratio) were also confirmed in an epidemiological study (54).

Putz et al. (53) described a mainly subclinical, asymptomatic small-fiber neuropathy, and mild impairment of cardiovascular autonomic function in IGT subjects. Similar to our present findings, HR variation and Valsalva ratio were decreased, whereas 30:15 ratio was unchanged among the tests evaluating parasympathetic activity; however, sympathetic function was also mildly impaired in patients with IGT (53). Moreover, these IGT patients also have abnormal circadian blood pressure regulation and increased diastolic blood pressure (56). Abnormal HR recovery was more common in patients with IFG (42%) and diabetes (50%) than in participants with normal glucose tolerance (31%) in a population-based Italian study; the relative

risks were 1.34 (95% confidence intervals = 1.2–1.5) and 1.61 (95% CI = 1.35–1.92), respectively (57).

Fasting plasma glucose found to be an independent predictor of abnormal HR recovery ($P < 0.0003$) even after adjustments for other confounders (57). Moreover, impaired glucose regulation significantly ($P < 0.006$) correlated with adrenergic autonomic dysfunction when age, an important confounder, was removed from the model (58). The self-assessment of autonomic symptoms by patients with IGT and early diabetes correlated to the degree of autonomic dysfunction defined by abnormal 30:15 ratio and reduced quantitative sudomotor axon reflex test sweat volume (59).

Limitations

Further clinical studies are warranted and needed to evaluate whether there is a direct link between the increased STV_{QT} detected in the present study and increased risk for sudden cardiac death in patients with IGT, preferably in a large patient cohort.

CONCLUSION

The present study is the first to show that short-term QT interval variability is higher in patients with IGT. The elevated temporal STV_{QT} and concomitant cardiac AN may serve as early indicators of the increased instability of cardiac repolarization and elevated risk for sudden cardiac death in patients with prediabetic states.

ETHICS STATEMENT

The studies described here were carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association and were approved by the Scientific and Research Ethical Committee of the Medical Research Council at the Hungarian Ministry of Health (ETT-TUKEB), under ethical approval No. 4987-0/2010-1018EKU (338/PI/010). All subjects have given written informed consent of the study.

AUTHOR CONTRIBUTIONS

The authors listed below gave the following contributions: IB, AN, TV, LB, GÁ, PK, JP, AV, and CL had substantial contributions to the conception of the work and design of the paper; AO, SN, AK, ZP, RT, LB, and CL contributed to the measurements and analyses of data; AO, IB, PK, JP, AV, and CL drafted the paper or revised it critically for important intellectual content. All authors (AO, IB, SN, AK, ZP, RT, AN, TV, LB, GÁ, PK, JP, AV, and CL) have read and approved the final manuscript.

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REVIEW
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Targeting Obesity and Diabetes to Treat Heart Failure with Preserved Ejection Fraction

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Heart failure with preserved ejection fraction (HFpEF) is a major unmet medical need that is characterized by the presence of multiple cardiovascular and non-cardiovascular comorbidities. Foremost among these comorbidities are obesity and diabetes, which are not only risk factors for the development of HFpEF, but worsen symptoms and outcome. Coronary microvascular inflammation with endothelial dysfunction is a common denominator among HFpEF, obesity, and diabetes that likely explains at least in part the etiology of HFpEF and its synergistic relationship with obesity and diabetes. Thus, pharmacological strategies to supplement nitric oxide and subsequent cyclic guanosine monophosphate (cGMP)—protein kinase G (PKG) signaling may have therapeutic promise. Other potential approaches include exercise and lifestyle modifications, as well as targeting endothelial cell mineralocorticoid receptors, non-coding RNAs, sodium glucose transporter 2 inhibitors, and enhancers of natriuretic peptide protective NO-independent cGMP-initiated and alternative signaling, such as LCZ696 and phosphodiesterase-9 inhibitors. Additionally, understanding the role of adipokines in HFpEF may lead to new treatments. Identifying novel drug targets based on the shared underlying microvascular disease process may improve the quality of life and lifespan of those afflicted with both HFpEF and obesity or diabetes, or even prevent its occurrence.

Keywords: metabolic disease, heart function, diastolic dysfunction, endothelial and microvascular dysfunction, inflammation, hypertension

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INTRODUCTION

Heart failure (HF) is a major public health problem on a global scale. Historically, HF was believed to originate from long standing systolic dysfunction, as assessed by reduced ejection fraction (HFrEF), and much progress has been made in the last several decades in slowing the inevitably fatal progression of this condition with drugs and in some cases implantable devices (1–5). However, nearly as many individuals are now recognized to exhibit signs of HF, namely dyspnea, fatigue, fluid retention, and exercise intolerance, but yet have a normal or near normal

ejection fraction (6–11). This condition of HF with preserved ejection fraction (HFpEF) is thought to be more common in women and more prevalent in the elderly, with similar mortality rates as HFrEF (12–15). HFpEF is documented as the leading cause of hospital admission in patients over 65 years of age and is predicted to be the leading cause of HF within a decade (16, 17). Notably, HFpEF is a leading cause of pulmonary hypertension (HTN) (18).

Diastolic dysfunction or impaired relaxation of the left ventricle (LV) is the common clinical condition of HFpEF and is attributable to both cardiac fibrosis and myofilament stiffness (19, 20). Contrary to expectations, recent clinical studies have failed to demonstrate the benefits offered by drugs effective in HFrEF to HFpEF patients (16, 21–23). Thus, HFpEF is one of the largest unmet needs in cardiovascular medicine, and there is a substantial requirement for new therapeutic approaches and strategies that target mechanisms specific for HFpEF (16). A general feature in HFpEF patients is the presence of several comorbidities (**Figure 1**) including HTN, anemia, atrial fibrillation (AF), obesity, and diabetes (7, 14, 16, 24–30). Moreover, comorbidities negatively affect prognosis to a greater extent in individuals with HFpEF than with HFrEF and have a greater impact on physical impairment as well (31). These observations support the proposition that aggressively targeting comorbidities may prove a more efficacious approach in the clinical management of HFpEF (32–34).

Approximately 50% of patients with HFpEF are obese (35), and HFpEF patients with an increased body mass index (BMI) $\geq$35 kg/m^2 are at an increased risk of an adverse outcome (death or cardiovascular hospitalization), independent of other key prognostic variables (36). Obesity is an identified risk factor for HFpEF (28, 37, 38). In a recent study on patients with HFpEF, Dalos et al. (39) found that one-third of patients over a 2-year follow-up reached the combined endpoint of HF hospitalization or cardiac death, which confirms the adverse prognosis

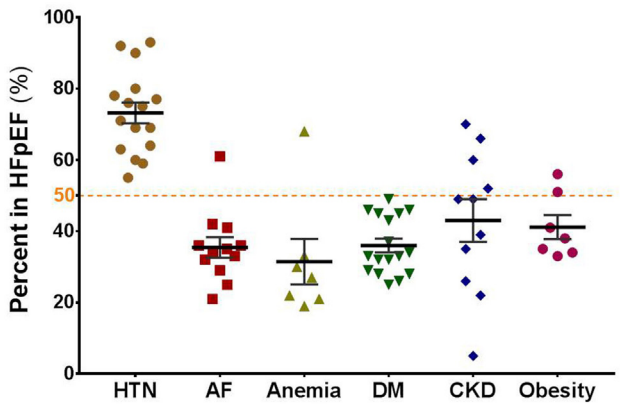

FIGURE 1 | Major comorbidities that negatively affect prognosis in patients with HFpEF. The graph shows the prevalence of comorbidities (in percent) in HFpEF patients enrolled in different clinical studies as summarized by Triposkiadis et al. (35): hypertension (HTN), atrial fibrillation (AF), anemia, diabetes mellitus or type II diabetes (DM), chronic kidney disease (CKD), obesity.

of HFpEF. NYHA class III or IV was a strong independent predictor of outcome, along with N-terminal pro-brain natriuretic peptide (NT-proBNP). Correlates of worse NYHA class included NT-proBNP, age, increased values for diastolic dysfunction, and diastolic pulmonary artery pressure. The most novel finding was that BMI was strongly associated with worse NYHA class. The investigators also concluded that a critical contributor to symptoms of breathlessness in patients with HFpEF is increased BMI. Obesity is likely more than a prominent comorbidity for HFpEF and critically involved in its pathogenesis. Increased adiposity promotes HTN, systemic inflammation, insulin resistance, and dyslipidemia, all of which are commonly observed in patients with HFpEF (40). Obesity also impairs cardiac, vascular, and skeletal muscle function (41, 42). Adipose tissue is metabolically active and produces inflammatory cytokines or adipokines, and a number of cardiovascular active substances. Growing evidence reveals that obesity-related microvascular dysfunction, which affects all organs, contributes to exercise-intolerance, and predisposes to the development of microvascular dementia, coronary microvascular angina, chronic obstructive pulmonary disease, pulmonary HTN, and chronic kidney disease (43).

Obesity and diabetes are present in HFpEF patients with a similar proportion (35, 44). In the absence of coronary artery disease and HTN, maladaptive cardiac remodeling associated with diabetes is properly referred to as diabetic cardiomyopathy (35, 45, 46). Accumulating evidence supports the notion that there are two distinct HF phenotypes associated with diabetic cardiomyopathy. Type 1 diabetes leads to HFrEF with a dilated left ventricular phenotype. In contrast, type 2 diabetes, which is a common outcome of obesity, is associated with HFpEF and concentric remodeling of the LV. Seferović and Paulus recently presented evidence attributing the etiology of the two phenotypes to the differential principal involvement of either microvascular endothelial cells (HFpEF) or cardiac myocytes (HFrEF) in the remodeling process (45). An ancillary study of the RELAX (Phosphodiesterase-5 Inhibition to Improve Clinical Status and Exercise Capacity in Diastolic Heart Failure) trial indicated that compared to non-diabetic HFpEF patients, those with diabetes were younger, more obese and more often male, with a higher prevalence of renal dysfunction, HTN, pulmonary disease, and vascular disease (47). Analysis of the I-Preserve [Irbesartan in heart failure with preserved ejection fraction (HFpEF)] trial showed that HFpEF patients with diabetes had more signs of congestion, worse quality of life, and a poorer prognosis with a higher risk of cardiovascular mortality and hospitalization (48). On the basis of 11 clinical features, HFpEF patients who were enrolled in the I-Preserve or CHARM-Preserved (effects of candesartan in patients with chronic HF and preserved left-ventricular ejection fraction) trials were found to fall into one of six subgroups; patients with obesity and or diabetes constituted a distinctive subgroup with (along with another subgroup characterized by advanced age) the worst event-free survival (49).

The goal of our review is to highlight developments in our understanding of obesity- and diabetes-related HFpEF achieved in the last five years. Given the broad magnitude, multifaceted, and

TABLE 1 | Potential targets or approaches for HFpEF.

Exercise and lifestyle modifications
Aerobic exercise training
Reduced calorie intake
Nitric oxide enhancement or replenishment
Nitroxyl donors
Inorganic nitrates/nitrites
β3 adrenergic receptor agonists
sGC stimulators
Endothelial cell mineralocorticoid receptor signal
Spironolactone
Non-coding RNAs
AngiomiRs
Glucose lowering drugs
Metformin
GLP-1 receptor agonists
SGLT-2 inhibitors
Novel approaches
• **Enhancing protective guanylyl cyclase systems**
LCZ696
PDE9 inhibitors
• **Independent of cyclic GMP**
$ProANP_{31-67}$

syndrome-like nature of the problem, this review is not intended to provide a comprehensive overview of obesity or diabetes and HFpEF. For instance, we do not discuss molecular signaling pathways in cardiac myocytes that are linked to hypertrophy, likely downstream of the initiating stress event (50), or that cause stiffness of myofilaments (51). We do not discuss signaling events in cardiac fibroblasts involved in collagen synthesis or turnover and fibrosis (52); nor do we deal with the importance of skeletal muscle abnormalities in HFpEF (53). Rather, we have chosen to focus on microvascular endothelial dysfunction, based on the compelling evidence that HFpEF is a manifestation of systemic vascular inflammation (54), before discussing potential pharmacological approaches (**Table 1**).

ROLE OF CORONARY MICROVASCULAR INFLAMMATION

Microvascular disease appears to be a common feature of obesity, type 2 diabetes, and HFpEF. It is now recognized that obesity is associated with chronic, low-grade systemic vascular inflammation that encompasses the coronary microvasculature and entails impaired angiogenesis, microvascular rarefaction, as well as endothelial dysfunction and impaired vasodilation due to reduced endothelial nitric oxide synthase (eNOS) activity (55–60). Increased circulating levels of adipokines and cytokines contribute to the inflammatory state (57, 59–61). Similarly, both macro- and microvascular derangements are prominent in patients with type 2 diabetes (62, 63), encompassing as well inflammation, endothelial dysfunction, hypercoagubility, functional disruption of the endothelium, rarefaction, and impaired angiogenesis. Also, individuals with type 2 diabetes mellitus suffer from a higher incidence of coronary heart disease as observed in obese patients (64–66).

Coronary microvascular inflammation is now postulated to play the key role in HFpEF progression, encompassing endothelial dysfunction and impaired nitric oxide (NO)-cyclic guanosine monophosphate (cGMP)—protein kinase G (PKG) signaling and increased collagen deposition (**Figure 2**) (54). Increased stiffness of both myofilaments and extracellular matrix is thought to impair diastolic function (15, 54, 67). The former is postulated to result from reduced PKG-mediated phosphorylation of titin (20, 54, 67), the protein that determines passive elasticity of cardiomyocytes, and the latter from increased collagen deposition and cross-linking (fibrosis) due to inflammatory endothelium-mediated recruitment of immune cells that activate resident cardiac fibroblasts (15, 20, 67, 68). Diastolic dysfunction is likely an antecedent event that interacts synergistically with other remodeling events at the cellular level to foster development of HFpEF. Recently, levels of inflammatory cells in endomyocardial biopsy samples from HFpEF patients were found to positively correlate with diastolic dysfunction (69) and coronary microvascular dysfunction was detected by angiography in patients with HFpEF (70). Further support for the involvement of myocardial microvascular inflammatory endothelial activation in the etiology of HFpEF comes from a study by Franssen et al. (71). These investigators reported that the myocardium of both HFpEF patients and an obesity-diabetic rat model of HFpEF showed upregulation of endothelial adhesion molecules, elevated expression of the pro-oxidant protein NOX2 in macrophages and endothelial cells but not cardiomyocytes, evidence of the uncoupling of eNOS, and reduced myocardial nitrite/nitrate concentration, cGMP content, and PKG activity.

Involvement of microvascular inflammation in HFpEF with the associated reduction in eNOS-mediated NO generation raises the possibility that enhancing cGMP-PKG signaling could be an efficacious therapeutic approach (46, 72). Potentially, this could be achieved with nitroxyl (HNO), the 1 electron-reduced congener of NO that has myocardial antihypertrophic and superoxide suppressing activity (73, 74), as well as anti-inflammatory actions on microvascular endothelial cell (75). Nitroxyl was also recently shown to inhibit TNF-induced endothelial cell and monocyte activation, as well as leukocyte adhesion to the endothelium, in isolated mouse aorta (76). Nitroxyl increases vasorelaxation and enhances cardiac contractility with positive inotropic and lusitropic effects due to a direct effect on cardiac myofilament proteins and enhancement of SERCA2a activity (77, 78). Nitroxyl may also substitute for NO in activating soluble guanylate cyclase (sGC) and increasing cGMP (79). Recently, chronic treatment with the nitroxyl donor 1-nitrosocyclohexyl acetate was found to attenuate left ventricular diastolic dysfunction in a mouse model of diabetes (80). Others have recently reported evidence indicating that inorganic nitrates and nitrites, which can be converted to NO in the body, are effective in alleviating some HFpEF symptoms (81–84). Lastly, both cardiac myocytes and endothelial cells express the third isotype of beta adrenergic receptors (β3 ARs), which couple to eNOS activation and anti-oxidant signaling (85, 86). Pre-clinical evidence suggests that β3 AR agonists, such as mirabegron, confer protection against diabetes-induced vascular dysfunction and may prove beneficial in HFpEF (85–88).

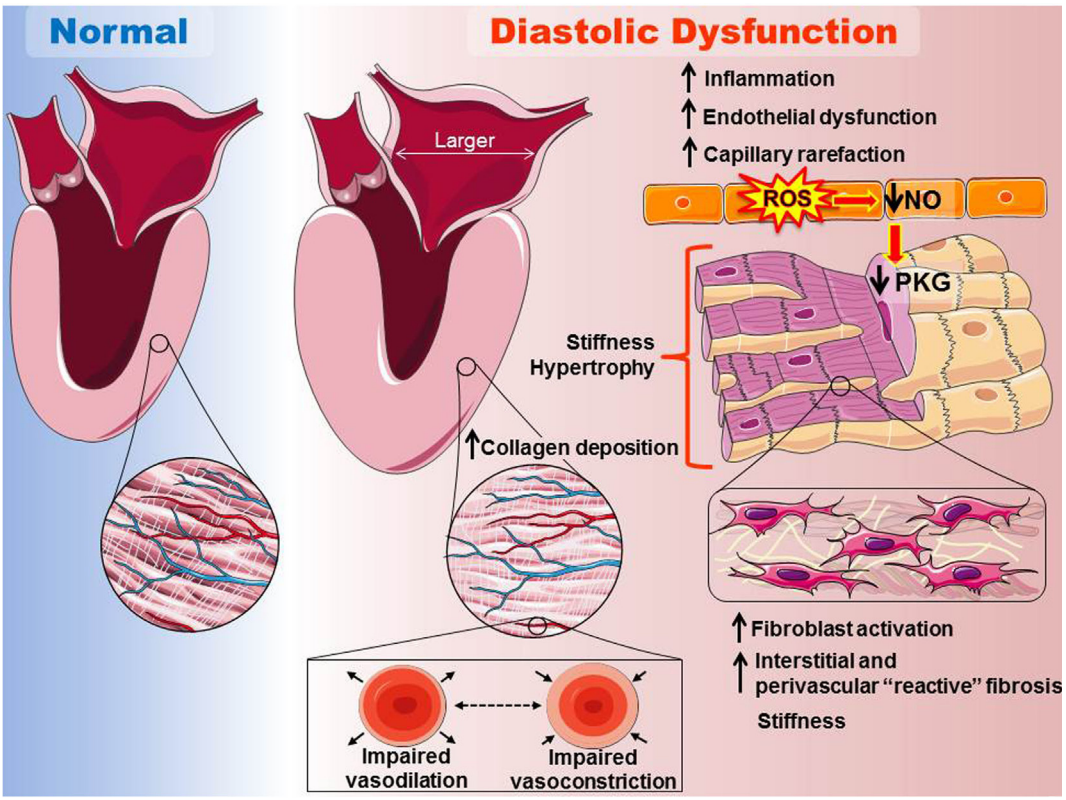

FIGURE 2 | Scheme for the proposed etiology of heart failure with preserved ejection fraction (HFpEF) relevant to obesity and type 2 diabetes. Left side: at the organ level, HFpEF is characterized by cardiac hypertrophy and a marked increase in the left ventricular mass/volume ratio (concentric remodeling), as well as increased stiffness and often enlargement of the left atrium. Right panel: coronary microvascular inflammation is postulated to play a key role in HFpEF progression, encompassing endothelial dysfunction and reduced nitric oxide (NO)-cyclic guanosine monophosphate (cGMP)—protein kinase G (PKG) signaling. Increased stiffness of both myofilaments and the extracellular matrix is thought to impair diastolic function of the heart. The former is postulated to result in part from reduced PKG-mediated phosphorylation of titin, the protein that determines passive elasticity of cardiomyocytes. The later would result from increased collagen deposition and cross-linking (fibrosis), due to loss of cGMP/PKG anti-fibrotic signaling and increased inflammatory endothelium-mediated recruitment of immune cells that activate resident cardiac fibroblasts. Diastolic dysfunction is likely an antecedent event that interacts synergistically with other remodeling events at the cellular level to foster development of HFpEF (images adapted and reproduced with permission from the copyright holder http://servier.com/Powerpoint-image-bank).

POTENTIAL TARGETS OR APPROACHES

Exercise and Lifestyle Modifications

Preclinical studies have demonstrated beneficial effects of exercise training to protect the heart in obese or diabetic animals. For instance, exercise was reported to protect the hearts of obese diabetic mice from ischemia-reperfusion injury (89) and to reverse cardiac microvascular rarefaction and impaired endothelium-dependent microvascular reactivity in obese diabetic rats (90). In patients with type 2 diabetes, exercise training was reported to improve brachial artery endothelial function (91), as well as to attenuate capillary rarefaction and improve microvascular vasodilator and insulin signaling (92). In contrast, Schreuder et al. (93) did not find any improvement in endothelial function after 8 weeks of training in type 2 diabetes patients. Although a reasonable supposition, there is insufficient data to assess whether dietary and lifestyle changes offer real promise to human sufferers of HFpEF. After all, exercise intolerance is a dominant symptom of HFpEF that contributes in a major way to reduced quality of life in these patients; plus, diabetes has the associated confounding factor of myocardial metabolic inflexibility (45). In a meta-analysis of randomized control trials, physical exercise was found to improve peak oxygen uptake and quality of life in HFpEF patients; however, no significant changes in LV systolic and diastolic function were noted (94). In older adults with type 2 diabetes and chronic renal insufficiency, a moderate protein diet showed long-term effects on low-grade inflammation, and oxidative stress (95), while in elderly HFpEF patients, exercise improved peak exercise oxygen consumption, although endothelial function or arterial stiffness were not altered (96). Among obese older patients with clinically stable HFpEF, caloric restriction or aerobic exercise training increased peak oxygen consumption, and the effects appeared to be additive (97); however, neither intervention had a significant effect on quality of life as assessed by the Minnesota Living with Heart Failure Questionnaire, suggesting that the patients may still have exhibited exertional dyspnea. In any event, no improvements in cardiac function were noted and improvements in peak exercise oxygen consumption were likely

due to non-cardiac peripheral adaptations (97, 98). Indeed, sarcopenic obesity may be a significant contributor to exercise intolerance in elderly HFpEF patients (99). Sustained and substantial weight loss *via* bariatric surgery, which was shown effective in improving left ventricular relaxation and reversing concentric LV remodeling and hypertrophy, might be considered to treat obesity-associated HFpEF in younger individuals; however, the long-term cardiovascular effects of this surgery in obese HFpEF patients would need to be assessed (33, 100).

In any case, acute exercise may serve as an important tool for detecting coronary microvascular dysfunction, which becomes more apparent when the heart is challenged in this manner (101). Additionally, exercise would cause the release of a number of hormones or cytokines in HFpEF patients that might impact on cardiac or microvascular function, an area of research that requires further exploration. Recently, exercise training was reported to increase ghrelin levels in patients with HFpEF, especially in patients with higher baseline adiponectin (102). Ghrelin is a gastric hormone that simulates appetite and is associated with weight gain. However, ghrelin was also reported to decrease blood pressure and increase cardiac output in health men (103) and to inhibit apoptosis of cardiomyocytes and endothelial cells *in vitro* (104). Levels of ghrelin are reduced in both obesity and type 2 diabetes (105). Irisin is a novel hormone (myokine) secreted by cardiac and skeletal myocytes in response to exercise that may regulate metabolism and limit weight gain, although its precise role is controversial (106, 107). Circulating levels of irisin are reported to be reduced or increased in obese subjects, but reduced in type 2 diabetic patients (106, 108, 109). Lower levels of irisin are associated with endothelial dysfunction (109, 110). Recently, irisin was found to improve endothelial function in obese mice *via* the activating 5′ adenosine monophosphate-activated protein kinase (AMPK)-eNOS pathway (110); in the spontaneously hypertensive rat, irisin-induced improvement in endothelial function, reduced blood pressure (111).

Endothelial Cell Mineralocorticoid Receptors Antagonism

Higher circulating aldosterone levels are observed in obesity (112) and type 2 diabetes (113). Moreover, aldosterone antagonism has proven effective in the clinical management of HFrEF (114, 115) and in attenuating cardiac dysfunction and maladaptive remodeling in pre-clinical animal models of obesity-associated HFpEF (116, 117). Surprisingly, the Treatment of Preserved Cardiac Function Heart Failure with an Aldosterone Antagonist (TOPCAT) study, a large randomized, double-blind clinical trial of spironolactone versus placebo in patients with symptomatic HFpEF, did not achieve a significant reduction in the primary composite outcome of time to cardiovascular death from cardiovascular causes, aborted cardiac arrest, or hospitalization for management of HF; however, TOPCAT did demonstrate that spironolactone decreases HF hospitalizations in HFpEF patients (118). Use of spironolactone for HFpEF was associated with an improvement in HF-specific health-related quality of life (119) and, in a separate study, improved exercise tolerance (120). Actually, the beneficial effects of spironolactone in HFpEF may be more significant. Subgroup analysis of TOPCAT by geographic region raised concerns about patient selection and dosing levels in the Russia/Georgia arm of the trial, whereas spironolactone was clearly superior to placebo in reducing cardiovascular events in the Americas (121). Also, spironolactone may have greater potential efficacy in HFpEF patients with lower ejection fraction (122) and, somewhat at odds with this, with lower levels of circulating natriuretic peptides and overall risk (123).

An endothelial-cell targeted strategy may optimize the beneficial actions of aldosterone antagonism in HFpEF. Based on accumulating evidence, Davel et al. recently proposed that in normal physiology, the endothelial mineralocorticoid receptor is vasoprotective; however, in the presence of cardiovascular risk factors, such as obesity and diabetes, endothelial mineralocorticoid receptor activation leads to endothelial dysfunction as a result of reduced eNOS activity and NO production, increased oxidative stress *via* eNOS uncoupling and NOX activation, as well as induced expression of adhesion molecules for inflammatory cells (124). Supporting this possibility is the observation that endothelial mineralocorticoid receptor deletion prevents obesity-induced diastolic dysfunction in female mice (125).

Non-Coding RNAs

MicroRNAs (miRNAs) are small non-coding RNAs (~21–25 nucleotides in length) that in animal cells generally bind to the 3′ UTR of mRNA to suppress gene expression by either transcript degradation or translational inhibition. The bloodstream contains multiple types of miRNAs in various types of vesicles and complexes, secreted from both healthy and dying cells of likely all tissues throughout the body (126). Since miRNA expression is dynamically regulated, circulating miRNAs are increasingly recognized as having potential utility for diagnostic and prognostic purposes. Recent reports have supported the diagnostic value of using circulating miRNA profiles to distinguish HF patients from non-HF controls and differentiating between HFrEF and HFpEF (127, 128). To date, miRNA profiles have not been defined for HFpEF patients on the basis of dominate comorbidity such as obesity or diabetes. However, both metabolic syndrome and type 2 diabetes are associated with altered circulating miRNA profiles (126, 129). The endothelium is a rich source of circulating miRNAs in both the healthy and disease states and the plasma mRNA profile provides an assessment of endothelial health (126). For instance, circulating and cardiac levels of pro-angiogenic miR-126 and miR-132 were found to be downregulated in type 2 diabetic individuals without any known history of cardiovascular disease (130). Decreased levels of these miRNAs were associated with cardiac microangiopathy as indicated by reduced capillaries and arterioles and increased endothelial cell apoptosis. Parallel findings in a mouse model of type 2 diabetes support the prognostic value of these "angiomiRs". Interestingly, swimming training in rats was reported to increase cardiac miRNA-126 expression and angiogenesis (131). Optimistically, the identification of particular miRNA signature in diabetes- or obesity-associated HFpEF could lead to miRNA-based therapies that use tissue-targeted exosomes to deliver anti-miRNA or miRNA mimics to treat microvascular dysfunction. Some of the challenges in making miRNA-based therapy a reality are discussed elsewhere (132–134).

An emerging area in cardiovascular medicine is the study of long non-coding RNAs (lncRNAs), which are transcripts larger than 200 nucleotides that control gene expression at the epigenetic, transcriptional, and posttranscriptional levels (135). Single-nucleotide polymorphisms which alter the expression of the lncRNA ANRIL (antisense non-coding RNA in the INK4 locus) are associated with coronary artery disease and type 2 diabetes (126). Recently, circulating levels of three lncRNAs were identified as biomarkers of diastolic function and remodeling in patients with well-controlled type 2 diabetes (136): long intergenic non-coding RNA predicting cardiac remodeling (LIPCAR), myocardial infarction-associated transcript (MIAT), and endothelial cell-enriched migration/differentiation-associated long non-coding RNA (SENCR). Although the cellular source was not defined in this study, LIPCAR is thought to originate from cardiomyocyte mitochondria, whereas MIAT and SENCR have been implicated in endothelial cell function/dysfunction, including inflammation and angiogenesis (126, 136, 137). The role and diagnostic/prognostic value of lncRNAs in obesity or diabetes associated HFpEF awaits investigation.

Glucose Lowering Drugs

The drug metformin has proven highly effective in the treatment of type 2 diabetes and is currently recommended as first line treatment. Metformin has beneficial actions by reducing hepatic glucose production and by activating AMPK, which enhances cellular glucose uptake. AMPK activation in cardiac myocytes may also inhibit hypertrophy (138). Preclinical studies demonstrated that AMPK activation by metformin restores endothelial function and NO bioavailability by attenuating oxidative and endoplasmic reticulum stress and by directly increasing eNOS activity (139, 140). However, metformin does not seem to improve LV stiffness in type 2 diabetic patients (141).

Concerns of increased adverse cardiovascular outcomes, including HF, are associated with the use of sulfonylureas and thiazolidinediones (TZDs) in diabetic patients (45, 142, 143). The situation with regard dipeptidyl peptidase-4 inhibitors is unsettled (144). Although glucagon-like peptide-1 (GLP-1) receptor agonists, liraglutide and semaglutide, showed a reduction in cardiovascular events, GLP-1 agonists do not seem to have a significant effect on natriuretic peptide levels in HF (45, 145). Much excitement has been generated by the recent approval of selective sodium glucose transporter 2 (SGLT-2) inhibitors, including empagliflozin, to treat type 2 diabetes. SGLT-2 inhibitors lower blood glucose by blocking sodium-dependent reabsorption of glucose in the proximal tubule and causing glycosuria. However, the beneficial actions of SGLT-2 inhibitors in type 2 diabetes seem to extend beyond glycemic control and are not completely understood (146). SGLT-2 inhibitors are associated with weight loss and reductions in blood pressure (without an increase in heart rate), visceral adiposity, plasma urate levels, and arterial stiffness/vascular resistance, as well as improvements in microvascular/macrovascular endothelial function and cardiac metabolism (146). The recently published results of the EMPA-REG OUTCOME trial revealed a marked reduction in deaths from cardiovascular causes, HF hospitalizations, and deaths from any cause when empagliflozin was added to standard care of patients with type 2 diabetes (147). At present, insufficient evidence precludes reaching a definitive conclusion as to whether the beneficial effects of empagliflozin represent a class effect of SGLT-2 inhibitors (148).

Novel Approaches That Enhance Guanylyl Cyclase Systems

Nitric oxide deficiency is postulated to be responsible for diastolic dysfunction in HFpEF patients due to impaired cGMP generation and PKG activation. Because of issues such as tolerance and preload reduction, organic nitrates seem not to be useful in treating HFpEF (149). Alternative ways of cGMP enhancement might hold more promise for future therapeutic benefit. sGC stimulators are a relatively new class of drugs that act *via* an allosteric site on sGC to synergize with NO in producing cGMP, thereby offsetting decreased NO due to diminished NO synthase activity (150). The recently completed phase II SOluble guanylate Cyclase stimulatoR in heArT failurE Study (SOCRATES) program consisted of two parallel studies to assess the potential utility of the sGC stimulator, vericiguat for treating HFrEF (SOCRATES-REDUCED) and HFpEF (SOCRATES-PRESERVED). The respective primary endpoints were change in NT-proBNP at 12 weeks, and change in NT-proBNP and left atrial volume at 12 weeks (151). Vericiguat was well tolerated; however, likely because of inadequate dosage level, SOCRATES-REDUCED yielded mixed, yet promising results (152). The outcome of SOCRATES-PRESERVED has not been reported but likely is complicated by the same shortfall in dosing as the SOCRATES-REDUCED study.

Alternative NO-independent ways to increase cGMP formation, which is linked to anti-hypertrophy and anti-fibrosis signaling in the heart (153, 154), may prove beneficial in treating HFpEF. Specifically, receptors for natriuretic peptides activate membrane-bound particulate GC. Indeed, several studies have shown favorable cardiorenal effects, including improvement of diastolic function, of exogenous supplementation of the natriuretic peptides, which are known to stimulate cGMP production in the heart, kidney, and vasculature (155, 156). In contrast, deletion of the BNP gene is characterized by diastolic dysfunction, cardiac remodeling, and rising of elevated blood pressure (157). A recently approved drug for the treatment of chronic HF, LCZ696 (brand name entresto), combines an angiotensin II type 1 receptor blocker (valsartan) with a neprilysin inhibitor (sacubitril). Sacubitril suppresses proteolysis of natriuretic peptides that enhance cGMP signaling independent of NO (158). The phase III study Efficacy and Safety of LCZ696 Compared to Valsartan, on Morbidity and Mortality in Heart Failure Patients With Preserved Ejection Fraction (PARAGON-HF) (NCT01920711) is currently underway, while preliminary data from the PARAMOUNT study have shown a significant reduction of the circulating levels of NT-proBNP (a major prognostic biomarker in HF) after 12 weeks of treatment, and an improvement of both cardiac size and New York Heart Association (NYHA) class at 36 weeks as compared to valsartan (159). Selective inhibitors of phosphodiesterase-9 (PDE9), which hydrolyzes natriuretic peptide-coupled cGMP and is upregulated in HFpEF, are another potential way to increase cardiac cGMP levels (160).

Inadequate processing and activation of natriuretic peptides appears to be a signature of HTN, resulting in an impaired counter-regulatory response of the natriuretic homeostatic control system (161, 162). Notably, although natriuretic peptides are useful to stratify HFpEF patients in conjunction with the NYHA classification system, circulating levels of BNP are not elevated as much in HFpEF patients as in HFrEF (163, 164). It is now established that elevated circulating natriuretic peptides in patients with overt cardiovascular diseases, although having a significant adverse prognostic value, are constituted mainly of biologically non-active forms, while mature active forms are virtually absent in severe congestive HF patients (165). In addition, obesity has a negative impact on the elevation of circulating levels of BNP as fatty tissue expresses the clearance receptor for the natriuretic peptide (NPRC) (163, 166). Therefore, supplementation of these cardioprotective natriuretic peptides may prove to be of therapeutic importance in obesity- or diabetes-associated HFpEF. Studies report that circulating atrial natriuretic peptide (ANP) can break down into multiple peptides, each of which has distinctive actions. One of these peptides, namely proANP$_{31–67}$, does not activate the cGMP pathway, but exerts a unique cardiac and renal protective response by increasing renal, as well as circulating levels of prostaglandin E2 (PGE2) (167–169). ProANP$_{31–67}$ also has vasodilatory actions and induces diuresis *via* inhibition of the basolateral Na^+–K^+ ATPase of the inner medullary collecting ducts resulting in increased Na^+ and renal water excretion (170, 171). Whether the potential benefits of proANP$_{31–67}$ extend to HFpEF is not established, although PGE2 has protective effects on the heart *via* enhancement of VEGF and eNOS expression levels and anti-inflammatory actions (172, 173). Future studies are warranted to determine whether the cardiorenal protective effects and the cardiac function enhancing properties of these hormones can be explained by mechanisms different from cGMP activation.

UNRESOLVED ISSUES

Adipose tissue is an endocrine organ that secretes multiple "adipokines" that have broad physiological and pathological impact throughout the body (174, 175). In obesity, the altered circulating adipokine profile contributes to systemic low-grade inflammation and the cardiovascular or obesity-related comorbidities defining the metabolic syndrome. Understanding the contribution of a particular adipokine to the disease process is a challenging task as the inflammatory milieu is a dynamic and fluid environment of multiple players with redundant or conflicting roles. A good case in point is the role of adiponectin in HFpEF. Adiponectin is the major adipokine produced by adipose tissue with anti-inflammatory, antidiabetic, anti-apoptotic, and anti-atherogenic properties (174, 176). Circulating adiponectin levels are decreased in obesity and type 2 diabetes and downregulation of adiponectin and its receptors is associated with insulin resistance and diabetes, as well as increased risk of HTN and coronary artery disease (174, 176). Animal studies have shown that adiponectin can inhibit cardiac hypertrophy and fibrosis, and reduce infarct size (174). Together these findings support the supposition that adiponectin might have therapeutic potential in HFpEF patients (176). However, circulating adiponectin levels are increased in both HFrEF and HFpEF (177). Furthermore, multiple studies have shown an association between higher adiponectin levels and increased mortality and cardiovascular disease mortality/morbidity in diverse populations (178). One confounding factor is that natriuretic peptides, which are elevated in HF due to hemodynamic stress and/or neurohormonal activation, may directly enhance adiponectin expression (178). Certainly, the question of which-time-point in the development and progression of HFpEF is an important consideration. Sex differences may play a role as well. Low adiponectin was associated with higher odds of indices of diastolic dysfunction in women, but lower odds in men, and lower adiponectin was associated with increased left ventricular mass only in women (179). Other variables that may come into play are adiponectin receptor desensitization, receptor subtypes, and the different-size molecular weight complexes of circulating adiponectin ("isoforms") (176).

PERSPECTIVES AND FUTURE DIRECTIONS

The role of sex as well as race in HFpEF, especially their interaction with comorbidities, is an evolving area of investigation. Early studies reported that HFpEF was more common among women than men (180, 181). Recently, the largest sex- and race-based subgroup analysis of HFpEF was published, involving data gathered from 1,889,608 hospitalizations (182). The study reported several noteworthy findings, including the following: (a) men with HFpEF were slightly younger than women with HFpEF and had a higher burden of comorbidities; (b) blacks with HFpEF were younger than whites with HFpEF, with lower rates of most comorbidities; (c) HTN, anemia, chronic renal failure, and diabetes, were more common among blacks; (d) AF was an important correlate of mortality only among women and blacks; and (e) with women, chronic pulmonary disease, and diabetes were more common among younger patients, but more common among older patients in men. Obviously, the influence of sex and race in the context of comorbidities to the heterogeneity of HFpEF is complicated and further study is needed. Another emerging area of interest is the additional classification according to the 2016 EC guidelines of HFmrEF, for HF patients exhibiting mid-range ejection fractions (183). The clinical profile, including comorbidities, and prognosis of patients diagnosed with HFmrEF, and the etiological and prognostic relationship of this HF phenotype to HFrEF and HFpEF needs to be addressed. The application of novel measures for assessing LV function such as strain imaging may be useful in this regard.

Obesity and diabetes are not only risk factors for the development of HFpEF but have significant impact on its symptoms and outcome. Therefore, focusing on these comorbid conditions in HFpEF might provide a novel therapeutic strategy. Coronary microvascular endothelial dysfunction with impaired NO-cGMP-PKG signaling is a shared condition that is thought to be the basis for diastolic stiffness, inflammation, oxidative stress, and maladaptive cardiac remodeling. Pharmacological

approaches that target this signaling axis offer promise in treating or preventing HFpEF. This would include: (a) NO replenishment (inorganic nitrates/nitrites), replacement (nitroxyl donors), or enhanced generation (β3 AR agonists and AMPK agonist); and (b) enhancers of NO-independent cGMP generation (LCZ696/entresto) or prevention of its breakdown (PDE9 inhibitors). A reappraisal of clinical results supports the utility of inhibiting the mineralocorticoid receptor in treating HFpEF, but additional study is warranted. In addition, given the pronounced side effects of spironolactone at higher doses, an endothelial cell-targeted approach might be judicious. miRNA and lncRNA profiling of HFpEF patients offers the promise of not only prognostic assessment and therapeutic monitoring, but personalized treatment strategies as well. A better understanding of the role of adipokines in obesity- and diabetes-associated HFpEF may open up new pharmacological avenues. Finally, SGLT-2 inhibitors offer great promise for treating or preventing HFpEF in obese and diabetic patients. A better understanding of the physiological and molecular basis for the cardiovascular protective actions of this new drug class should foster the development of even more effective compounds.

AUTHOR CONTRIBUTIONS

All authors contributed conceptually to the manuscript. All authors authored sections of the manuscript, contributed to figure design, and approved the final version. All appropriate permissions have been obtained from the copyright holders of any work that has been reproduced in this manuscript.

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In Vivo and *In Vitro* Analysis in Coronary Artery Disease Related to Type 2 Diabetes

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Aim: The leading cause of morbidity and mortality in patients with type 2 diabetes mellitus (DM) is coronary artery disease (CAD), a condition often asymptomatic but severe in these patients. Although glucose metabolism impairment and oxidative stress are known actors in the endothelial dysfunction/remodeling that occurs in diabetic patients, the relationship between cardiovascular disorders and DM is not fully understood. We have performed both an *in vivo* imaging and *in vitro* molecular analysis to investigate diabetic-specific CAD alterations.

Methods: Computed tomography coronary angiography (CTCA) was performed in a group of 20 diabetic patients with CAD (DM^+CAD^+), 20 non-diabetic with CAD (DM^-CAD^+), 10 diabetic non-CAD patients (DM^+CAD^-), and 20 non-diabetic healthy subjects (HS). Imaging quantitative parameters such as calcium score (Cascore), calcified plaque volume (CPV), non-calcified plaque volume (NCPV), total plaque volume (TPV), remodeling index (RI), and plaque burden were extracted for each CAD subject. Moreover, the expression levels of superoxide dismutase 2 (SOD2) and liver X receptor alpha (LXRα) genes were analyzed in the peripheral blood mononuclear cells, whereas hyaluronan (HA) concentrations were evaluated in the plasma of each subject.

Results: Imaging parameters, such as Cascore, CPV, RI, and plaque burden, were significantly higher in DM^+CAD^+ group, compared to DM^-CAD^+ ($P = 0.019$; $P = 0.014$; $P < 0.001$, $P < 0.001$, respectively). SOD2 mRNA was downregulated, while LXRα gene expression was upregulated in DM^+CAD^-, DM^+CAD^+, and DM^-CAD^+ groups compared to HS ($P = 0.001$, $P = 0.03$, and $P = 0.001$ for SOD2 and $P = 0.006$, $P = 0.008$, and $P < 0.001$ for LXRα, respectively). Plasmatic levels of HA were higher in DM^-CAD^+, DM^+CAD^-, and DM^+CAD^+ groups, compared to HS ($P = 0.001$ for the three groups). When compared to DM^-CAD^+, HA concentration was higher in DM^+CAD^- ($P = 0.008$) and DM^+CAD^+ ($P < 0.001$) with a significant difference between the two diabetic groups ($P = 0.003$). Moreover, HA showed a significant association with diabetes ($P = 0.01$) in the study population, and the correlation between HA levels and glycemia was statistically significant ($\rho = 0.73$, $P < 0.001$).

Conclusion: In our population, imaging parameters highlight a greater severity of CAD in diabetic patients. Among molecular parameters, HA is modulated by diabetic CAD-related alterations while SOD2 and LXRα are found to be more associated with CAD but do not discriminate between diabetic and non-diabetic subgroups.

Keywords: type 2 diabetes, coronary artery disease, computed tomography coronary angiography, biomarkers, atherosclerosis

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INTRODUCTION

Type 2 diabetes mellitus (DM) is the most important risk factor for the onset of coronary artery disease (CAD), causing glucose metabolism impairment and endothelial dysfunction mediated by oxidative stress and inflammation (1). A complex network of signaling pathways is involved in these pathological processes leading to the development and progression of cardiac dysfunction. In response to myocardial damage, the heart undergoes a progressive anatomical and functional transformation known as "remodeling" (2).

Several imaging modalities have been used to detect CAD in diabetic patients including invasive coronary angiography, myocardial scintigraphy and dobutamine stress echocardiography (3). Even if invasive coronary angiography is the gold standard for identifying obstructive lesions, it only depicts the lumen of the vessel, greatly underestimating the burden of atherosclerosis (4). Myocardial scintigraphy and dobutamine stress echocardiography highlight perfusion defects (inducible ischemia and necrosis), but they lack a direct visualization of coronary arteries (5). Unlike these, computed tomography coronary angiography (CTCA) is a powerful diagnostic tool to rule out CAD thanks to its high negative predictive value (6). It allows quantification of atherosclerotic burden providing comprehensive information about the location, severity, and features of coronary atherosclerotic plaques and can be useful for risk stratification (7, 8).

Atherosclerosis is a multistage pathological condition involving an imbalanced lipid metabolism and immune response leading to a chronic inflammation of the arterial wall with the formation of the atherosclerotic plaque and consequent thickening of vessel wall and lumen stenosis (9, 10).

The first step of atherosclerosis is endothelial dysfunction; atherosclerotic lesions initiate in regions characterized by low shear stress resulting in an increase of adhesiveness of circulating monocytes to the vessel wall and subendothelial accumulation of low-density lipoprotein (LDL) (9). Common cardiovascular risk factors, such as smoking, diabetes, hypertension, and hypercholesterolemia, are causes of dysfunction endothelial (10). The LDL particles in the intima are susceptible to oxidation by reactive oxygen species or other enzymes released from inflammatory cells. Oxidized LDL triggers the expression of adhesion molecules and the secretion of chemokines by endothelial cells that drive the intimal infiltration by immune cells forming the so-called "fatty streaks" especially consisting of monocyte-derived macrophage-like foam cells. Subsequently, vascular smooth muscle cells migrate and proliferate into the site of lesion producing an excessive amount of connective tissue with the consequent formation of the fibroatheromatous plaque leading to thickening of vessel wall and stenosis of coronary lumen (9–11). One of the major issues in CAD diagnosis and management is that symptoms onset in the advanced state of disease. Indeed, most individuals show no manifestations for long time before the first onset of symptoms, often with a fatal event.

Oxidative stress is a key component in the development and progression of DM and its vascular complications such as CAD (12, 13). The onset and progression of CAD involves multiple cell types, and whole-blood gene expression profiling has the potential to provide information about dynamic changes in disease states and on underlying disease mechanisms (14).

Superoxide dismutase 2 (SOD2) is one of the major antioxidant defense systems against free radicals (15). Mutations or polymorphisms of SOD2 gene are associated with DM progression and complications, where the reduction of total antioxidant capacity and depletion of plasma antioxidants could be related to induced-oxidative stress damage (16–21).

Nuclear liver X receptors (LXR) comprise liver X receptor alpha (LXRα) and liver X receptor beta (LXRβ), which are key regulators of macrophage function, controlling transcriptional programs involved in lipid homeostasis and inflammation. The inducible LXRα is highly expressed in macrophages, liver, adrenal gland, intestine, adipose tissue, lung, and kidney, whereas LXRβ is ubiquitously expressed (22). LXRs are involved in the regulation of cholesterol metabolism fundamental for the pathogenesis of CAD and inhibit atherogenesis, inflammation and autoimmune reactions (22). Furthermore, an additional role of LXRs is to contribute to glucose homeostasis, demonstrating potent glucose-lowering and insulin-sensitizing effects (23, 24). Despite extensive research in the field of LXR biology, however, very little is known about the regulation of expression and activity of these receptors.

Hyaluronan (HA) is present in low amount in normal blood vessels but increases in vascular diseases as well as in DM (25). It seems to have an important role in diabetic angiopathy (26–28) and is associated with an increased risk for developing CAD also in non-diabetic patients (29). HA is increased in vascular plaques, and its high metabolism causes their destabilization (30). Furthermore, the fragmentation of HA triggers inflammatory processes and activates leukocytes to produce superoxide radical causing oxidative stress (31).

To date, studies integrating parameters calculated by CTCA and biological markers in DM patients have been focused on the association between CTCA findings (mostly coronary artery calcium) and biological markers of inflammation (IL-6, IL-1β, TNF-α, hs-CRP, and YKL-40) and endothelial dysfunction (sVCAM-1, sICAM-1, and sICAM-3) (32–36). There are no data about the association between imaging parameters and gene expression profiling in DM.

In this study, we have analyzed the three above mentioned molecular markers that underlie important steps of the atherosclerotic process: endothelial dysfunction, oxidative stress, lipid homeostasis, and inflammation. In this regard, we have analyzed SOD2 and LXRα gene expression and HA plasmatic concentrations in a group of 20 diabetic patients with known CAD (DM^+CAD^+), 20 non-diabetic patients with CAD (DM^-CAD^+),

Abbreviations: DM, diabetes mellitus; CAD, coronary artery disease; HS, healthy subjects; DM^+CAD^-, diabetic non-CAD patients; DM^+CAD^+, diabetic patients with CAD; DM^-CAD^+, non-diabetic patients with CAD; CTCA, computed tomography coronary angiography; HU, Hounsfield unit; Cascore, calcium score; CPV, calcified plaque volume; NCPV, non-calcified plaque volume; TPV, total plaque volume; RI, remodeling index; SOD2, superoxide dismutase 2; LXRα, liver X receptor alpha; HA, hyaluronan; NSP, number of coronary artery segments with plaque; NCS, number of coronaries with significant stenosis.

10 diabetic non-CAD patients (DM^+CAD^-), and 20 non-diabetic healthy subjects (HS). Furthermore, the purpose of our study was to investigate diabetic-specific CAD alterations using both quantitative imaging parameters derived from CTCA and molecular biomarkers.

MATERIALS AND METHODS

Patient Recruitment

Computed tomography coronary angiography was performed in 20 DM^+CAD^+ patients, 20 DM^-CAD^+ patients, 10 DM^+CAD^- patients, and 20 HS referred to our institution for suspected CAD. All clinical characteristics such as laboratory parameters, presence of cardiovascular risk factors, and medical history were accurately recorded.

Diabetes was defined as treatment with drugs or fasting blood glucose ≥126 mg/dL. Dyslipidemia was defined as treatment with drugs or fasting serum total cholesterol ≥240 mg/dL, or LDL cholesterol ≥140 mg/dL, or high-density lipoprotein cholesterol <40 mg/dL, or triglyceride ≥150 mg/dL. Hypertension was defined as treatment with drugs or systolic blood pressure (SBP) ≥140 mmHg or diastolic blood pressure (DBP)≥ 90 mmHg. Anthropometrical measurements including body weight and height were recorded and body mass index (BMI) was calculated. Blood pressure and resting heart rate were measured after ≥5 min rest with a sphygmomanometer. Physical activity in HS and patients was evaluated according to the WHO guidelines for adults 18–65 aged; specifically the performance of at least 150 min of moderate-intensity aerobical physical activity per week [50–70% of maximum heart rate (MHR)] or at least 75 min of vigorous-intensity aerobic physical activity throughout the week (70–80% MHR) (37). None of the recruited subjects had physical disabilities.

Patients with known history of cancer, cardiomyopathy, active infections, chronic or immune-mediated diseases, renal failure, hepatic failure, and not suitable for cardiac imaging (atrial fibrillation, arrhythmia, or pre-scan heart rate greater than 65 bpm) were excluded from the study to avoid confounding effects due to other variables.

Sample Collection

Peripheral venous blood samples were collected after a 12 h overnight fasting immediately before i.v. cannulation for CTCA examination. All tubes were centrifuged within 30 min of collection at 1,900 *g* for 10 min at 4°C to separate plasma and cellular components. Aliquots of plasma were transferred into cryostat tubes and stored at −80°C until analysis. PBMNCs were isolated by Ficoll gradient using HISTOPAQUE-1077 (Sigma Diagnostics, MO, USA) and frozen at −80°C until total RNA extraction. All biological samples were stored at the IRCCS SDN Biobank (38). The study and the protocol were approved and reviewed by the institutional ethics committee (IRCCS Fondazione SDN, protocol no. 7-13). The study was performed in accordance with the ethical standards of the institutional ethics committee and with the Helsinki Declaration. A written informed consent was obtained from all subjects enrolled.

CT Angiography Protocol and Image Analysis

Computed tomography coronary angiographies were performed on a CT scanner (Discovery CT750 HD, GE Healthcare), with a 64 mm × 0.625 mm collimation, 350 ms rotation time, and 228 ms temporal resolution. A prospective ECG-triggered scan without contrast medium was used for calcium score (Cascore) evaluation followed by a retrospective scan with ECG tube current modulation. Contrast enhancement was obtained by a bolus tracking technique with scan starting when a region of interest placed in the ascending aorta at the pulmonary bifurcation reached a threshold of 150 Hounsfield unit (HU). Contrast material (iomeprol 400 mg I/mL, Iomeron 400, Bracco, Milan, Italy) was injected at 5–6 mL/s through an 18-gauge intravenous antecubital catheter and was followed by saline solution at the same flow. Tube voltage and contrast agent volume were adapted to patient anatomical features such as BMI, calcifications, or stents. Images were reconstructed with a section thickness of 0.625 mm and an increment of 0.4 mm; standard and sharp reconstruction filter kernels were used; an additional sharper convolution kernel was used in patients with stents or calcification. The best data set was chosen according to the phase of the cardiac cycle with lower artifacts and coronary motions. Images were sent to a dedicated offline workstation (GE Advantage workstation 4.6, GE Healthcare) where MIP, cMPR, and 3D volume rendering were generated. Cascore was calculated by the SmartScore tool to obtain the Agatston score. Total plaque volume (TPV), non-calcified plaque volume (NCPV), calcified plaque volume (CPV), and total lumen volume were measured for the major coronaries using the HU cutoff values reported in Ref. (39). The resulting values were summed to determine a per-patient plaque volume. Total vessel volume was determined summing TPV and total lumen volume. Plaque burden was obtained dividing TPV by total vessel volume (40). The remodeling index (RI) was calculated by dividing the cross-sectional vessel area at the site of maximum luminal narrowing including plaque by the cross-sectional vessel area in the most proximal atherosclerotic free segment chosen as reference (41). The total number of coronary artery segments exhibiting plaque (NSP) was determined according to the modified American Heart Association 16-segment classification (42) for each patient (less or more than 8 segments affected). Significant coronary stenosis was defined as a decrease in the luminal diameter of >50% in one or more of the major coronary arteries; the total number of coronaries (NCS) with significant stenosis was calculated for each patient (less or more than one stenotic vessel). All scans were analyzed by two experienced, independent radiologists; therefore, a consensus interpretation was arrived to obtain a final coronary CT diagnosis according to the international SCCT guidelines (43).

RNA Extraction and Reverse Transcription

Total RNA was isolated from PBMCs using TRIzol Reagent (Thermo Fischer Scientific, USA) as previously described (44). The quantity and quality of RNA were measured using the NanoDrop 1000 (Thermo Fischer Scientific, USA). Total RNA (0.5 μg) was reversed transcribed (RT) using the SuperScript® III

First-Strand Synthesis SuperMix for qRT-PCR (Thermo Fischer Scientific, USA) according to the protocol of the manufacturer. The RT was performed using the Bio-Rad iCycler Thermal Cycler with the following protocol: incubation at 25°C for 10 min (primer annealing), 42°C for 30 min (cDNA synthesis), and 85°C for 5 min (termination of cDNA synthesis). Immediately after, the samples were cooled down and stored at −20°C.

Quantitative Real-time PCR

The optimal reference genes for the study were selected as previously reported (45). Gene expression was quantified on the MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Primers pairs were designed through OLIGO 6.7 program, and their specificity was verified with the BLAST program for test of sequence homology, a test for secondary structures and optimization of multiplex setup. All primers were purchased from Life Technologies. All samples were run in triplicate for genes of interest and reference genes using 1 µL of cDNA and iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, USA) in a 25 µL final volume reaction. The thermal profile employed was 3 min of initial step of denaturation at 95°C followed with denaturation for 15 s at 95°C, annealing at 60°C for 30 s, and elongation at 72°C for 30 s for 40 cycles. Melt curve analysis was performed to verify a single product species. Relative expression (fold change) was calculated by the $2^{-\Delta\Delta CT}$ method (46). Mean and SE were determined by averaging relative expression levels across three independent experiments, each determined in triplicate.

HA Measurement

Plasmatic levels of HA were determined by enzyme-linked immunosorbent assay (ELISA) using Quantikine Hyaluronan Immunoassay kit (DHYAL0) (R&D Systems, Abingdon, UK), in accordance with the protocol supplied by the manufacturer. Briefly, samples were incubated with HA binding protein coated on microplates for 2 h at room temperature. After incubation, the microplates were washed five times with wash buffer, and further incubated with 100 µL of peroxidase labeled HA binding protein for 2 h at room temperature. After incubation, the microplates were again washed five times, and further incubated with 100 µL of peroxidase substrate for 30 min at room temperature in a dark room. The reaction was stopped by the addition of 100 µL of stop solution. The optical density of each well was determined using a microplate reader set to 450 nm within 30 min. HA concentration in each sample was calculated using the standard curve obtained with the purified HA solutions, included in the kit as references.

Statistical Analysis

Statistical analysis was performed using R Core Team (version 3.03 Austria, Vienna). Continuous variables were expressed as mean ± SD or as median (1 quartile and 3 quartile). Data were tested for normality through the Shapiro–Wilk test and for homoscedasticity through the Levene test. For comparison between two groups, *t*-test was used if gaussianity was met; otherwise the Mann–Whitney *U* test was chosen. For comparison among four groups, the one-way analysis of variance was used if both gaussianity and homoscedasticity were met; otherwise the Kruskal–Wallis test was chosen. In case of statistical significance, the Tukey–Kramer test and the Conover test were used for multiple comparisons as parametric and non-parametric test, respectively. Categorical variables were expressed as percentage and were compared using the Fisher's exact test. The Spearman correlation test was performed to assess linear relationship between variables; in case of binary variables, the association was tested by the Wilcoxon rank sum test. A $P < 0.05$ was considered for statistical significance (rounded to the third decimal place).

RESULTS

Clinical Characteristics of Study Groups

The baseline demographic and clinical characteristics of the study population are summarized in **Table 1**. Heart rate was significantly different between HS and DM^+CAD^+ ($P < 0.01$) and HS and DM^-CAD^+ ($P < 0.01$) since only 10% of HS was in treatment with beta blocker agents, while no statistical significance was found between both CAD groups and DM^+CAD^-. Considering the metabolic markers, glycemia was significantly higher in DM^+CAD^+ and DM^+CAD^- patients compared to HS ($P < 0.01$ and $P < 0.001$, respectively) and DM^-CAD^+ subjects ($P < 0.01$ and $P < 0.001$, respectively). Of diabetic patients, in DM^+CAD^+ group, 16% were insulin users, 64% were in treatment with antihyperglycemic agents, and 20% were not in treatment; in DM^+CAD^- group, the percentage of treatments were, respectively, 10% for insulin, 80% for antihyperglycemic drugs, and 10% were not treated. Total cholesterol, LDL- and HDL-cholesterol plasmatic concentrations did not significantly differ among the four groups, reflecting the effects of statin therapy to which 73.68% of DM^+CAD^+, 50% of DM^-CAD^+, 40% of DM^+CAD^-, and 5% of HS were subjected. Furthermore, SBP and DBP were not statistical different among the groups ($P = 0.50$ and $P = 0.52$, respectively). In this regard, hypertensive patients were in treatment with beta blocker agents ($P = 0.008$), calcium channel blockers ($P = 0.68$), and ACE inhibitors ($P = 0.06$).

Imaging Parameters

There was significant difference between DM^-CAD^+ and DM^+CAD^+ according to NCS and NSP ($P = 0.026$, $P = 0.04$, respectively). Cascore was significantly higher in DM^+CAD^+ compared to DM^-CAD^+ (**Figures 1** and **2**): 1,068.7 (517.2–2,086.85) vs 214.05 (72.98–970.15) $P = 0.019$. As regards plaque characterization, CPV was significantly higher in DM^+CAD^+ [105.85 (51.2–341.73) mm^3] compared to DM^-CAD^+ [42 (7.2–105.9) mm^3] $P = 0.014$, but there was no significant difference according to NCPV and TPV: 519.85 (411.93–1,064.85) mm^3 for DM^+CAD^+ and 421.85 (240.10–689.58) mm^3 for DM^-CAD^+ $P = 0.37$ and 688.95 (470.05–1,436) mm^3 for DM^+CAD^+ vs 454.45 (257.78–820.83) mm^3 for DM^-CAD^+ $P = 0.16$, respectively. RI was 1.40 ± 0.24 for DM^+CAD^+ and 1 ± 0.19 for DM^-CAD^+ $P < 0.001$, and plaque burden was 0.45 ± 0.14 for DM^+CAD^+ and 0.27 ± 0.15 for DM^-CAD^+ $P < 0.001$. Results are summarized in **Table 2**.

In our population, RI highly correlated with plaque burden ($\rho = 0.65$, $P < 0.001$). Cascore showed a positive correlation with

TABLE 1 | Clinical parameters of patients and healthy subjects.

Clinical parameters	HS	DM^+CAD^-	DM^+CAD^+	DM^-CAD^+	*P* value[a]
Age	58 ± 8.37	60.8 ± 13.5	61.24 ± 10.47	64.4 ± 9.33	0.07
BMI	26.9 ± 3.62	28.94 ± 4.2	29.36 ± 5.43	27.73 ± 3.14	0.07
SBP (mmHg)	119.38 ± 11.16	122 ± 14.7	126.25 ± 19.20	118.75 ± 7.91	0.50
DBP (mmHg)	75.63 ± 6.23	77 ± 5.81	74.67 ± 10.36	78.75 ± 3.54	0.52
Heart rate (bpm)	67.86 ± 11.09	60.20 ± 6.15	56.00 ± 6.50	54.68 ± 6.49	0.005
Ejection fraction (%)	57.50 ± 3.54	55.78 ± 6.07	51.46 ± 9.90	56.00 ± 5.00	0.41
Glycemia (mg/dL)	93.71 ± 10.26	126.90 ± 14.43	132.43 ± 26.15	97.90 ± 12.17	0.006
Azotemia (mg/dL)	36.91 ± 7.44	37.8 ± 9.15	39.84 ± 16.04	38.48 ± 11.84	0.71
Creatinine (mg/dL)	0.88 ± 0.17	1 ± 0.19	1.03 ± 0.19	1.04 ± 0.17	0.05
Sex (M)	60%	60%	70%	75%	0.50
CAD familiarity	45%	40%	60%	60%	0.51
Smoke	25%	30%	15%	35%	0.75
Hypertension	45%	70%	75%	55%	0.04
Dyslipidemia	35%	70%	75%	45%	0.05
Physical activity	25%	20%	200%	25%	0.80
Total cholesterol (mg/dL)	187.35 ± 31.55	160.7 ± 72.45	160.75 ± 65.95	172.80 ± 47.31	0.51
LDL-c (mg/dL)	133.86 ± 26.47	87.24 ± 38.45	83.50 ± 68.59	107.60 ± 43.71	0.31
HDL-c (mg/dL)	55.25 ± 19.55	48.27 ± 15.50	48.50 ± 12.40	41.40 ± 8.02	0.45
Tryglycerides (mg/dL)	123.57 ± 52.99	135.28 ± 55	150.40 ± 66.54	119.25 ± 53.21	0.70
Medical treatments					
Beta blocker agents (%)	10%	40%	52.63%	52.63%	0.008
Calcium channel blockers (%)	20%	10%	10.53%	21.05%	0.68
ACE inhibitors (%)	15%	30%	42.11%	21.05%	0.06
Statins (%)	5%	40%	73.68%	50%	0.008
Antiplatelets agents (%)	5%	10%	78.95%	52.63%	<0.001
Diabetic medications					
Oral hypoglicemic (%)		80%	64%		0.04
Insulin (%)		10%	16%		0.23
No treatment (%)		10%	20%		0.05

[a]*Comparison among HS, DM^+CAD^-, DM^+CAD^+, and DM^-CAD^+.*

SBP, systolic blood pressure; DBP, diastolic blood pressure; CAD, coronary artery disease; LDL, low-density lipoprotein; BMI, body mass index.

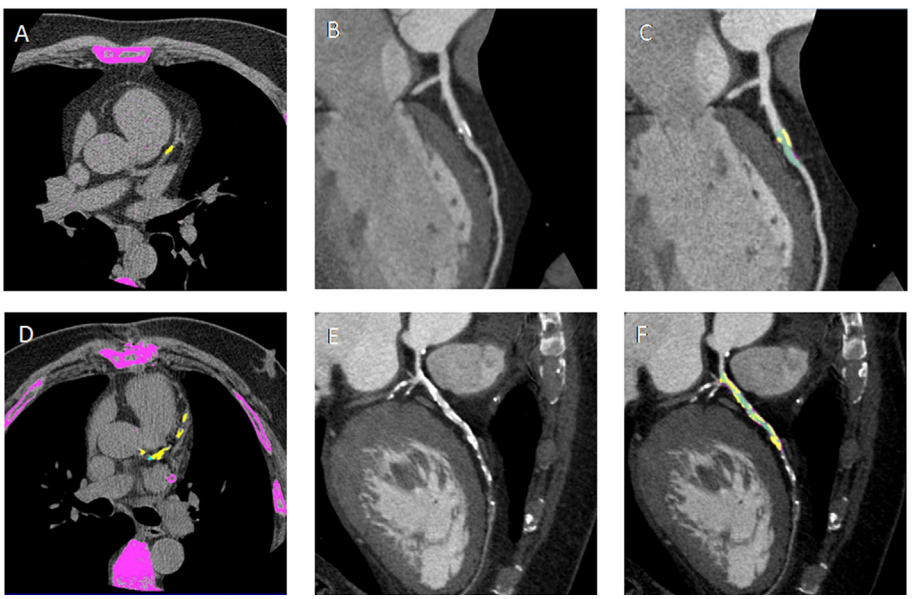

FIGURE 1 | **(A,D)** Non-contrast enhanced images showing calcium deposits (yellow) on the left descending coronary artery (LAD) in a non-diabetic CAD patient (DM^-CAD^+) and in a diabetic CAD patient (DM^+CAD^+), respectively. **(B,E)** cMPR of LAD is provided for DM^-CAD^+ and DM^+CAD^+. **(C,F)** Plaque characterization: the calcific (yellow) and non-calcific (pink) components of the plaque are highlighted; the vessel lumen is represented in green. DM^+CAD^+ displayed significantly higher coronary calcium values compared to DM^-CAD^+.

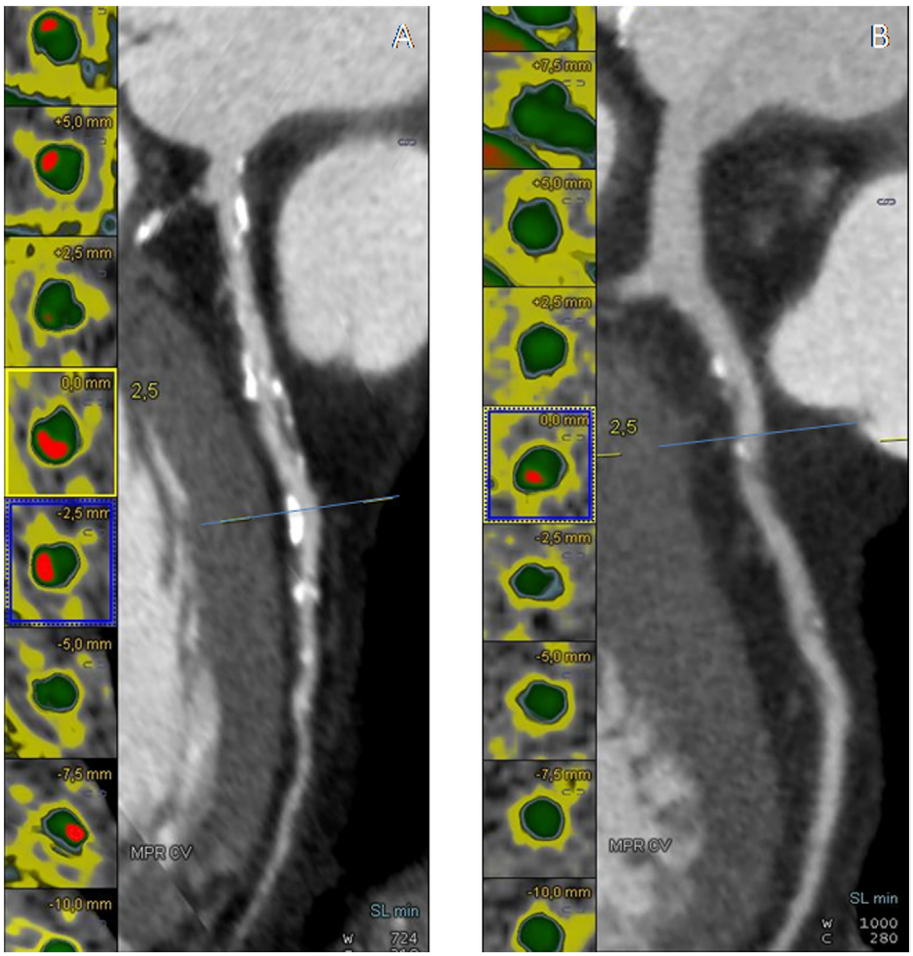

FIGURE 2 | **(A,B)** Cross-sectional view and cMPR of the left descending coronary artery (LAD) in a non-diabetic CAD patient (DM^-CAD^+) and in a diabetic CAD patient (DM^+CAD^+). In cross-sectional images, the vessel lumen is represented in green whereas the calcific component of the plaque is red.

TABLE 2 | Imaging parameters.

Imaging parameters	DM^+CAD^+	DM^-CAD^+	*P* value[a]
Number of coronaries with stenosis	44.4%[c]	10%[c]	0.026
NSP[b]	78%[d]	45%[d]	0.04
Calcium score[b]	1,068.7 (517.2–2,086.85)	214.05 (72.98–970.15)	0.019
Calcified plaque volume (mm^3)[b]	105.85 (51.2–341.73)	42 (7.2–105.9)	0.014
Non-calcified plaque volume (mm^3)[b]	519.85 (411.93–1,064.85)	421.85 (240.10–689.58)	0.37
Total plaque volume (mm^3)[b]	688.95 (470.05–1,436)	454.45 (257.78–820.83)	0.16
Remodeling index	1.40 ± 0.24	1 ± 0.19	<0.001
Plaque burden	0.45 ± 0.14	0.27 ± 0.15	<0.001

[a]*Comparison among DM^+CAD^+ and DM^-CAD^+.*
[b]*Data are expressed as median (1 quartile–3 quartile).*
[c]*Patients with >1 coronary stenotic vessels.*
[d]*Patients with >8 coronary segments exhibiting plaque.*

NCPV ($\rho = 0.83$, $P < 0.001$), CPV ($\rho = 0.96$, $P < 0.001$), TPV ($\rho = 0.88$, $P < 0.001$), and plaque burden ($\rho = 0.60$, $P < 0.001$). Moreover, a significant correlation was found between plaque burden and NCPV ($\rho = 0.57$, $P < 0.001$), CPV ($\rho = 0.64$, $P < 0.001$), and TPV ($\rho = 0.60$, $P = 0.001$).

Gene Expression Profiling

We evaluated, by quantitative real-time PCR, SOD2 and LXRα gene expression in PBMCs from our population (**Table 3**). For both genes, ΔCT was computed and compared between the four groups. Molecular analysis showed that SOD2 mRNA

TABLE 3 | Molecular parameters.

Molecular parameters	HS	DM^+CAD^-	DM^+CAD^+	DM^-CAD^+	*P* value[a]
Superoxide dismutase 2	2.36 ± 2.61	5.70 ± 3.28	4.57 ± 3.56	5.75 ± 3.04	0.009
Liver X receptor alpha	4.94 ± 2.14	2.77 ± 1.36	3.27 ± 1.79	2.15 ± 1.16	0.005
Hyaluronan	46.90 ± 23.79	105.56 ± 18.13	120.74 ± 21.17	90.05 ± 35.11	<0.001

[a]*Comparison among HS, DM^+CAD^-, DM^+CAD^+, and DM^-CAD^+.*

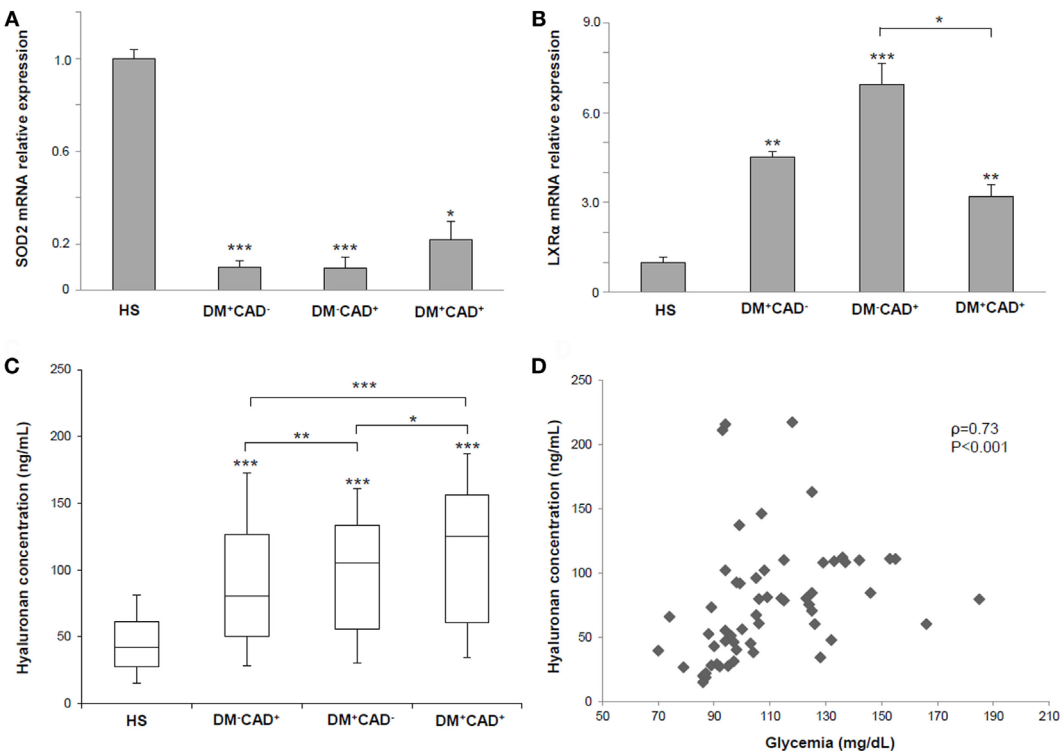

FIGURE 3 | **(A)** Superoxide dismutase 2 (SOD2) mRNA relative expression in PBMNCs of healthy subjects (HS), diabetic non-CAD patients (DM^+CAD^-), non-diabetic CAD patients (DM^-CAD^+), and diabetic CAD patients (DM^+CAD^+). **(B)** Liver X receptor alpha (LXRα) gene expression in PBMNCs of HS, DM^+CAD^-, DM^-CAD^+, and DM^+CAD^+. **(C)** Median plasma hyaluronan (HA) concentrations (ng/mL) in HS, DM^+CAD^-, DM^-CAD^+, and DM^+CAD^+ (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). When not specified, significance is referred to the comparison vs HS. **(D)** Correlation between HA levels and glycemia in HS, DM^+CAD^-, DM^-CAD^+, and DM^+CAD^+ patients ($\rho = 0.73$, $P < 0.001$).

was downregulated in DM^+CAD^- (ΔCT = 5.70 ± 3.28; fold change = 0.10 ± 0.03; $P = 0.001$), DM^+CAD^+ (ΔCT = 4.57 ± 3.56; fold change = 0.22 ± 0.08; $P = 0.03$), and DM^-CAD^+ (ΔCT = 5.75 ± 3.04; fold change = 0.10 ± 0.05; $P = 0.001$) compared to HS (ΔCT = 2.36 ± 2.61), with no statistically significant difference between the two CAD groups (with and without DM) (**Figure 3A**). LXRα gene expression was significantly upregulated in DM^+CAD^- (ΔCT = 2.77 ± 1.36; fold change = 4.51 ± 0.20 $P = 0.006$), DM^+CAD^+ (ΔCT = 3.27 ± 1.79; fold change = 3.19 ± 0.42; $P = 0.008$), and DM^-CAD^+ (ΔCT = 2.15 ± 1.16; fold change = 6.93 ± 0.70; $P < 0.001$) compared to the HS (ΔCT = 4.94 ± 2.14), with a significant difference between the two CAD groups ($P = 0.03$) (**Figure 3B**). No statistically significant correlation was found between SOD2 and Cascore ($\rho = -0.04$, $P = 0.81$), NCPV ($\rho = -0.03$, $P = 0.85$), and TPV ($\rho = -0.02$, $P = 0.89$) as well as between LXRα and Cascore ($\rho = 0.13$, $P = 0.44$), NCPV ($\rho = 0.10$, $P = 0.55$), and TPV ($\rho = 0.10$, $P = 0.57$).

Comparison of HA Levels

In the HS group, mean concentration of plasma HA was 46.90 ± 23.79 ng/mL. Compared with HS, HA concentrations were higher in DM^-CAD^+ (90.05 ± 35.11 ng/mL; $P = 0.001$), DM^+CAD^- (105.56 ± 18.13 ng/mL; $P = 0.001$), and DM^+CAD^+ (120.74 ± 21.17 ng/mL; $P = 0.001$) (**Figure 3C**). When compared to DM^-CAD^+, HA concentration was significantly higher in DM^+CAD^- ($P = 0.008$) and DM^+CAD^+ ($P < 0.001$) with a significant difference between two diabetic groups ($P = 0.003$). Correlation of HA levels with Cascore, NCPV, and TPV revealed $\rho = 0.29$, $P = 0.076$, $\rho = 0.30$, $P = 0.073$, and $\rho = 0.31$, $P = 0.063$, respectively.

Risk Factors and Molecular Markers Analysis

Analysis on risk factors and molecular data showed no significant association between sex and SOD2, LXRα, and HA ($P = 0.85$;

$P = 0.21$; $P = 0.75$, respectively) as well as regarding familiarity ($P = 0.83$; $P = 0.64$; $P = 0.55$, respectively) and smoke ($P = 0.73$; $P = 0.17$; $P = 0.49$, respectively). Furthermore, no significant correlations were found between BMI and the three molecular parameters (SOD2 $\rho = 0.12$, $P = 0.35$; LXRα $\rho = -0.11$, $P = 0.35$; HA $\rho = 0.19$, $P = 0.15$). HA was significantly correlated with age ($\rho = 0.46$, $P < 0.001$), unlike SOD2 ($\rho = 0.03$, $P = 0.97$) and LXRα ($\rho = 0.03$, $P = 0.95$). HA showed also a significant association with dyslipidemia ($P = 0.01$) and diabetes ($P = 0.01$) in the study population, while statistical analysis on hypertension revealed a $P = 0.08$. On the other hand, SOD2 and LXRα were not significantly associated with the previously mentioned risk factors (SOD2 vs dyslipidemia $P = 0.45$, SOD2 vs hypertension $P = 0.63$, SOD2 vs diabetes $P = 0.47$, LXRα vs dyslipidemia $P = 0.85$, LXRα vs hypertension $P = 0.53$, and LXRα vs diabetes $P = 0.61$). Correlation between HA levels and glycemia was statistically significant ($\rho = 0.73$, $P < 0.001$) (**Figure 3D**), while no significant correlation was found between SOD2 ($P = 0.62$) and LXRα ($P = 0.55$) gene expression and glycemia.

DISCUSSION

In this study, we have exploited an imaging and molecular based analysis to investigate diabetic-specific CAD alterations in selected groups of patients.

Calcium score, CPV, plaque burden, and RI were significantly higher in DM^+CAD^+ compared to DM^-CAD^+. Previous studied have examined CAD and plaque features in diabetic patients by CTCA. Diabetics showed extensive coronary artery calcium deposits and, therefore, a larger atherosclerotic plaque burden with a consequent higher risk for all-cause mortality than in non-diabetic patients (5, 47–54). Gao et al. (47) found that diabetics compared to non-diabetics have higher total coronary artery calcium, a higher proportion of coronary segments with plaque and multivessel obstructive disease. In a study by Van Werkhoven et al., obstructive CAD and the number of diseased segments, with obstructive and non-obstructive plaques, were higher in diabetics than non-diabetics. Total Agatston score was higher in diabetic patients (440 ± 786 vs 195 ± 404, $P < 0.001$) (5). Khazai et al. found that segment involvement score, segment stenosis score, and total plaque score were higher in diabetics but there was no significant difference in the number of non-calcified plaque between the two groups (50). In one study by Pundziute et al., diabetics showed more diseased segments and more segments with non-obstructive CAD, but Agatston score was similar between the two groups (54). Furthermore, Chu et al. detected more calcified plaques than mixed or non-calcified plaques in diabetics. Among the different degrees of stenosis, mild narrowing was most common, and no significant difference between non-obstructive stenosis and obstructive stenosis was observed (48). In agreement with the aforementioned works, in our study, DM^+CAD^+ presented more diseased coronaries in terms of coronary calcium, significant stenoses, atherosclerotic burden, and extent of disease. Furthermore, we have quantified RI in diabetic patients by CTCA providing an additional prognostic value comparable only to invasive procedures such as intravascular ultrasound (55, 56). A recent study analyzed CAD features comparing hypertensive, dyslipidemic, and diabetic patients by CTCA reporting the prevalence of positive remodeling as a qualitative parameter (57).

Positive coronary arterial remodeling is a compensatory enlargement of coronary arterial lumen in response to atherosclerotic plaque formation. Histopathological studies proved that positive remodeling is associated with infiltration of inflammatory cells, expression of pro-inflammatory cytokines, and increased protease activity (58, 59). Positive remodeling is associated with vulnerable plaque and progression of atherosclerosis. High plaque burden, together with positive remodeling, means more prone to rupture plaques in diabetic patients and, therefore, a worse prognosis and a major likelihood of cardiac event occurrence.

In the last decade, a great amount of data demonstrated a complex interaction between blood cells and the arterial wall with the consequent activation of oxidative and inflammatory pathways, leading to the development of CAD.

Our results showed that the expression levels of SOD2 gene were reduced in CAD patients compared to HS, while no significant difference was found between diabetic and non-diabetic CAD subjects. Previous studies reported controversial findings for the effect of SOD2 activity relative to CAD. A recent study by Peng et al. (60) showed that plasmatic concentration of SOD1 and SOD2 was higher in CAD than in healthy control. Our findings were in line with a gene expression study performed by Abdullah et al. (61) showing a downregulation of this gene in PBMCs of angiographically confirmed CAD patients (≥50% stenosis). These data indicate that SOD2 might serve as surrogate biomarker for CAD.

Data from *in vitro* and *in vivo* models have demonstrated a key role of LXRα in the regulation of processes involved in CAD and DM such as inflammation and glucose homeostasis (62, 63). Our findings reported that LXRα gene expression was significantly upregulated in DM^+CAD^+ and DM^-CAD^+ compared to HS. Although previous study by Dahlman et al. (64) investigated the association of LXRα and DM, we demonstrated also a differential expression of this gene between DM^+CAD^+ and DM^-CAD^+ groups suggesting this parameter as a possible biological hallmark for diabetic condition. HA plasmatic concentrations showed significant difference between diabetic and non-diabetic patients with higher values in patients affected by both DM and CAD suggesting a possible additive detrimental effect on endothelial dysfunction. A significant positive correlation was found between HA levels and glycemia in our study population. Our findings were in line with previous studies, also reporting a critical role for HA in DM-related atherosclerosis (26–29, 65). In vascular dysfunction, HA triggers smooth muscle cells' dedifferentiation, which contributes to vessel wall thickening. Furthermore, HA is able to modulate inflammation by altering the adhesive properties of endothelial cells. In hyperglycemic conditions, HA accumulates in vessels and can contribute to the diabetic complications in macro- and microvasculature (25).

However, no study has yet examined the relationship between HA levels and vascular function assessed by CTCA. Our data suggested that serum HA levels positively correlated with poor glycemic control and angiopathy and, due to the pivotal role in favoring atherogenesis, this molecule could be used as a surrogate marker of vascular function.

In vitro molecular analysis represents a promising tool to stratify patients for CAD risk, while *in vivo* CTCA analysis is able to identify and characterize selective diabetic coronary features. These results gain clinical relevance, considering that most patients referred to elective invasive coronary angiography for CAD suspicious are not found to have obstructive CAD (66, 67). In patients with molecular alterations suggestive for CAD, we demonstrated by CTCA specific changes of coronary plaques in diabetic patients. Moreover, recently, this imaging technique has been used to evaluate its long-term prognostic value among patients with diabetes mellitus compared with non-diabetic subjects (68).

Nevertheless, our study has some limitations: the reduced sample size has influenced the statistical power; therapeutic treatments could have affected our results; a more accurate analysis with different genomic/proteomic techniques, on a wider pool of *in vitro* markers is needed to deeply investigate molecular and imaging phenotypic interplay in diabetic CAD patients. The analyzed biomarkers are not myocardial specific CAD molecules but can be downregulated or upregulated in blood also in presence of atherosclerotic processes involving peripheral arteries and/or supra aortic vessels. Moreover, recent studies have demonstrated that diabetes can be considered a CAD equivalent condition, independently from the clinical/imaging evidences of pathology (69, 70), determining the choice of specific diabetic-related CAD biomarkers attractive.

CONCLUSION

This study suggests an imaging and molecular based analysis to investigate cardiovascular alterations in diabetic patients. CTCA imaging parameters highlight a greater severity of CAD in diabetic patients. Among molecular parameters, HA is modulated by diabetic CAD-related alterations while SOD2 and LXRα are found to be more associated with CAD rather than to diabetes. Further studies are needed to better characterize the pathology and identify more specific biomarkers, also considering the complex multifactorial pathophysiology of CAD in diabetic patients.

ETHICS STATEMENT

The study was approved and performed in accordance with the ethical standards of the institutional ethics committee (IRCCS Fondazione SDN, protocol no. 7-13) and with the Helsinki Declaration. Written informed consent was obtained from all subjects for being included in the study.

AUTHOR CONTRIBUTIONS

CC designed and supervised the study. TI and EF recruited subjects, performed the experiments and data analysis, and wrote the manuscript. MA, MS, and CC reviewed the manuscript. All the authors read and approved the final manuscript and agreed to its submission.

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