CARDIOPROTECTION, SEX AND GENDER DIFFERENCES

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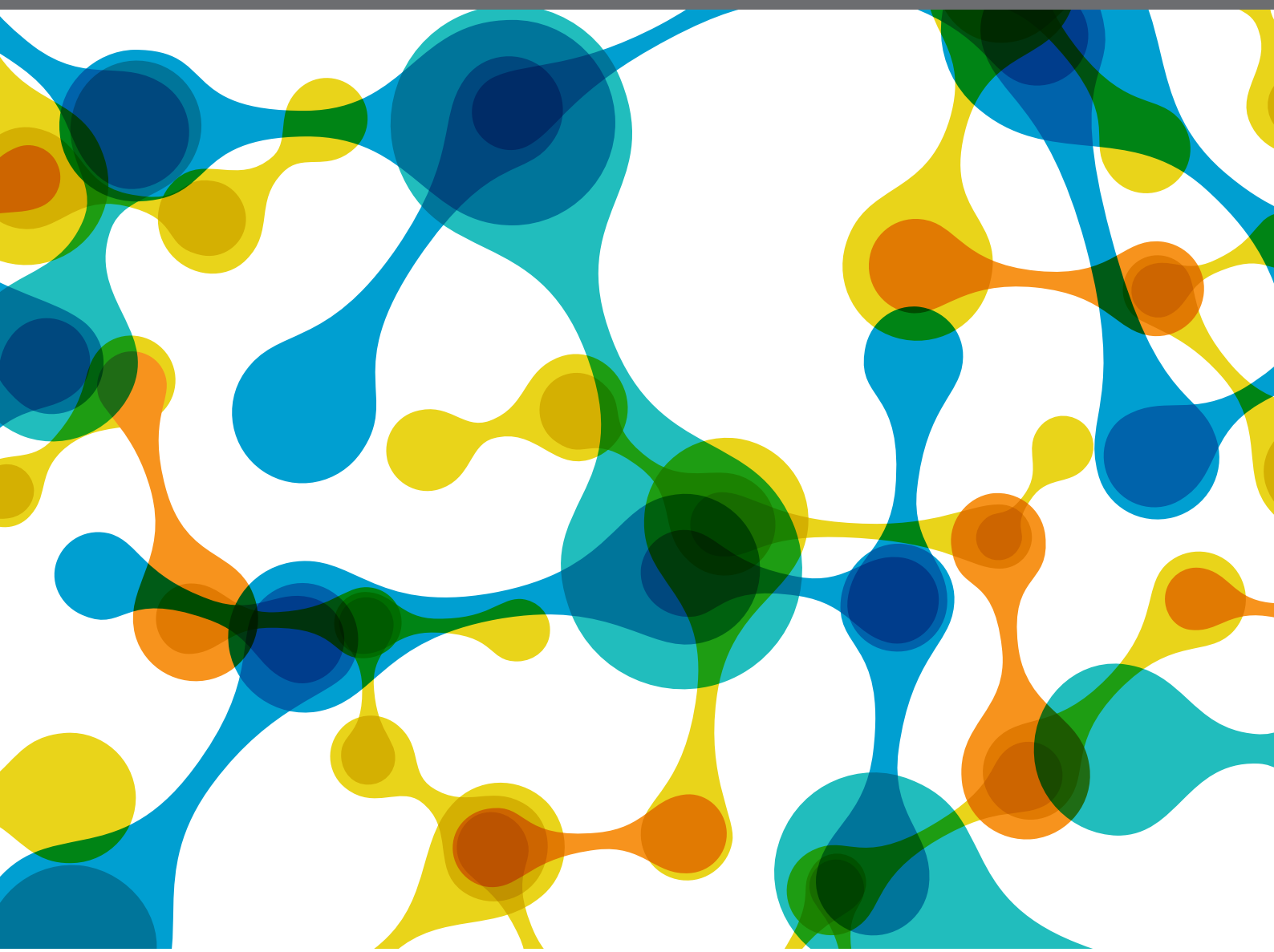

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CARDIOPROTECTION, SEX AND GENDER DIFFERENCES

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Editorial: Cardioprotection, sex and gender differences

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Editorial on the Research Topic Special Issue
Cardioprotection, sex, and gender differences

The special issue, "Cardioprotection, sex and gender differences" focuses on various aspects of sex and gender and supports that they play a significant role in cardiovascular diseases (CVD). It has long been known, and supported by numerous studies, that sex differences play a major role in cardiac susceptibility to cardiovascular disease. Indeed, important and relevant disparities in pathophysiology, clinical presentation and management were observed between men and women. o date, the numerous molecular mechanisms underlying these differences are currently still partially unknown. It is important to underline the distinction between the two terms, sex and gender. While "sex differences" are merely due to biological differences, "gender differences" depend on many aspects, including the environment, lifestyle and characteristics of attitude. The use of experimental models and a careful analysis of clinical data is currently emerging that both disparities show fundamental importance both in the diagnosis and management of cardiovascular diseases. Therefore, gender differences may be considered a fundamental branch of precision medicine.

In the present special issue, these topics have been covered with both original works and reviews (Akther et al.; Leutner et al., 2021; Li et al.; Liu et al.; Xu et al.; Ytrehus et al., 2021; Yu et al., 2022). Forrny et al. analyzed these differences in the setting of the metabolic syndrome. The Authors focused their attention on type 2 diabetes, a chronic disease associated with micro and macrovascular complications. Indeed, the Authors reviewed the literature and reported an increased risk of CVD in women with diabetes compared to men, in particular concerning the risk of coronary heart disease accompanied by higher mortality in case of acute myocardial ischemia.

Another interesting aspect is reported by Querio et al. in a mini review on the response to cardioprotective maneuvers in different experimental models related to sex-dependent response. The Authors underline the influence of sex on the outcome of cardioprotective procedures. When applied, cardioprotection significantly reduces damage from ischemia/reperfusion. Within their review, Querio and collaborators highlighted that the protective

maneuvers show effects that are not always positive when applied in female experimental models of a given age. The noticeable differences in response to these procedures are partly due to sex hormones, some of which decrease over the life span in women. The presence of sex hormones, in particular estrogen, has a highly protective role against ischemia/reperfusion damage. In this report Querio et al. describe the molecular pathways involved in cardioprotective protocols, clarifying at least in part how sex hormones can help improving physiological responses to CVD (Querio et al., 2021).

Ueda et al. discuss the significance of sex differences in the pathogenesis of cardiovascular disease. The Authors, through an overview of the results of the clinical studies obtained to date, relating to sex differences and hormone replacement therapy. The recent pandemic condition has highlighted important disparities in the pathogenesis of COVID, as reported by numerous studies and reviews (Pagliaro and Penna, 2020; Penna et al., 2020; Viveiros et al., 2021). In this regard, in relation to COVID19, in this special issue Cheng et al. found in a retrospective cohort study that the incidence of myocardial damage in patients with COVID-19 is sex-dependent, predominantly in association with a higher degree of inflammation and bleeding disorders in men. The paper reports the results of a retrospective study conducted on 1,157 COVID-19 patients (49.4% female and 50.6% male) who were hospitalized in Huoshenshan hospital from 12 March 2020 to 11 April 2020. The Authors emphasize the protective role played by sex hormones, in particular with regard to the inflammatory reaction and the state of coagulation. The latter, varying based on gender and women's specific protective mechanisms, likely mediated by sex differences in the incidence of myocardial damage. Sex differences are maintained in the incidence of adverse outcomes in COVID-19 patients.

Another aspect related to a component of the COVID-19 scenario was presented by (Yu et al., 2022). The Authors analyzed the role of the angiotensin converting enzyme 2 (ACE 2) in the hypertensive heart. The results obtained indicate the presence of a male preponderance for an increase in the gene expression of ACE and ACE2. The results are in agreement with the role of androgens or male chromosomal complement in controlling the expression of the two ACE genes.

In conclusion, the studies published in this special issue confirm the importance of hormonal balance in determining CVD, an aspect that has also been apparent during the COVID-19 pandemic.

Author contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of interest

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

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Leutner, M., Matzhold, C., Kautzky, A., Kaleta, M., Thurner, S., Klimek, P., et al. (2021). Major depressive disorder (MDD) and antidepressant medication are overrepresented in high-dose statin treatment. *Front. Med. (Lausanne)* 8, 608083. Erratum in: Front Med (Lausanne). 2021 Apr 07;8:677866. doi:10.3389/fmed.2021.608083

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ORIGINAL RESEARCH
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Major Depressive Disorder (MDD) and Antidepressant Medication Are Overrepresented in High-Dose Statin Treatment

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Objective: To examine the dose-dependent relationship of different types of statins with the occurrence of major depressive disorder (MDD) and prescription of antidepressant medication.

Methods: This cross-sectional study used medical claims data for the general Austrian population ($n = 7,481,168$) to identify all statin-treated patients. We analyzed all patients with MDD undergoing statin treatment and calculated the average defined daily dose for six different types of statins. In a sub-analysis conducted independently of inpatient care, we investigated all patients on antidepressant medication (statin-treated patients: $n = 98,913$; non-statin-treated patients: $n = 789,683$). Multivariate logistic regression analyses were conducted to calculate the risk of diagnosed MDD and prescription of antidepressant medication in patients treated with different types of statins and dosages compared to non-statin-treated patients.

Results: In this study, there was an overrepresentation of MDD in statin-treated patients when compared to non-statin-treated patients (OR: 1.22, 95% CI: 1.20–1.25). However, there was a dose dependent relationship between statins and diagnosis of MDD. Compared to controls, the ORs of MDD were lower for low-dose statin-treated patients (simvastatin>0–<=10 mg:OR: 0.59, 95% CI: 0.54–0.64; atorvastatin>0–<=10 mg:OR:0.65, 95%CI: 0.59–0.70; rosuvastatin>0–<=10 mg:OR: 0.68, 95% CI: 0.53–0.85). In higher statin dosages there was an overrepresentation of MDD (simvastatin>40–<=60 mg:OR: 2.42, 95% CI: 2.18–2.70, >60–80 mg:OR: 5.27, 95% CI: 4.21–6.60; atorvastatin>40–<=60 mg:OR: 2.71, 95% CI: 1.98–3.72, >60–<=80 mg:OR: 3.73, 95% CI: 2.22–6.28; rosuvastatin>20–<=40 mg:OR: 2.09, 95% CI: 1.31–3.34). The results were confirmed in a sex-specific analysis and in a cohort of patients taking antidepressants, prescribed independently of inpatient care.

Conclusions: This study shows that it is important to carefully re-investigate the relationship between statins and MDD. High-dose statin treatment was related to an overrepresentation, low-dose statin treatment to an underrepresentation of MDD.

Keywords: statins, depression, dyslipidemia, dosage, precision medicine

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INTRODUCTION

Statins rank among the most-prescribed drugs worldwide and have significant lipid-lowering effects and hence they are usually prescribed to prevent and treat cardiovascular disease (CVD). Recently published guidelines of the European Society of Cardiology (ESC/EAS) recommend that high-risk patients, such as patients with type-2 diabetes with organ damage or patients with CVD, should have low-density lipoprotein cholesterol (LDL-cholesterol) levels lower than 55 mg/dl (1). The main mechanism of statins is inhibition of 3-hydroxy-3-methyl-glutaryl-coenenzyme A reductase (HMG-CoA reductase), which in turn inhibits synthesis of mevalonic acid, the main substrate for the synthesis of cholesterol. Cholesterol itself is the basic substance for the synthesis of essential hormones such as sex hormones or vitamin D. In this context, earlier studies have shown that statins can lower the concentrations of sex hormones for instance (2–5).

A connection between sex hormones and depression has also been demonstrated by earlier studies. Low levels of estrogen have been associated with depression in women (6–10) and withdrawal of hormone therapy for remitted perimenopausal depression has been linked to recurrence of depressive symptoms (7). Similar results have been shown for low testosterone levels in men (11–13). Earlier studies report associations between low levels of cholesterol and consequently statin usage and depression, as well as related symptoms such as lowered mood, aggression and suicidality (14–17). A potential mechanism for these associations is impaired serotonin signaling, as cholesterol is required for serotonin 1A receptor function (18–20) and hence downregulation of the serotonin 1A receptor has been linked to mood disorders such as depression (21).

More recently, the neuroinflammation hypothesis for depression has gained traction, also pointing to antidepressant effects of anti-inflammatory agents such as statins (22). Along these lines, some studies report a protective effect of statins on the development of depression (22–29). One of the major problems of the existing literature is that especially the relationship between high-dose statin treatment and MDD has yet to be investigated in detail. Data on the relationship between high-dose statin treatment and MDD in randomized controlled trials (RCTs) are particularly sparse and hence the existing literature does not clearly demonstrate that statins have an antidepressant effect, which is the main reason why they are not considered in antidepressant therapy (30). Given the paucity of data, the aim of the present study was to investigate the relationship between statins of different types and dosages and MDD.

METHODS

Study Design

This cross-sectional retrospective analysis investigated medical claims data for the general Austrian population. Two groups [patients (1) with and (2) without statin treatment] were compared in order to investigate the relationship between statin treatment and MDD.

Patient Population

In the present analysis, health data were investigated for all Austrians receiving health care services, i.e., ∼97% of the population. These data include all diagnoses recorded during a hospital stay and data for all drug prescriptions exceeding a prescription charge of EUR 4.70. All patients alive during the observational period from January 2006 to December 2007 ($n = 7{,}945{,}775$) were analyzed, and age and sex were noted. Patients born in these years or aged >90 were excluded. The final study cohort consisted of 7,481,168 patients (3,507,903 males; 3,973,265 females). Medical prescriptions during the study period were analyzed using the Anatomical Therapeutic Chemical (ATC) Classification System codes.

Characterization of Patients With MDD

We identified all patients diagnosed with MDD during hospital stays by using primary and secondary diagnoses, as defined by the International Classification of Diseases, 10th revision (ICD-10), codes. We classified patients as having MDD if they had a primary or secondary diagnosis of F32 (major depressive disorder, single episode) or F33 (major depressive disorder, recurrent). In order to strengthen our results, we performed a sub-analysis in which we investigated all patients who had been prescribed antidepressants ($n = 888{,}596$), irrespective of inpatient hospital stays. In another sub-analysis, we considered only patients with at least one hospital stay ($n = 2{,}011{,}334$), i.e., for whom diagnoses could in principle have been recorded.

Characterization of Statin-Treated Patients and the Control Group

The statin-treated group consisted of patients prescribed one of the following six statins in at least four different quarters of a year (representing the common prescription procedure in Austria and patients' compliance) during the observational period: simvastatin (ATC-code:C10AA01), lovastatin (ATC-code:C10AA02), pravastatin (ATC-code:C10AA03), fluvastatin (ATC-code:C10AA04), atorvastatin (ATC-code:C10AA05), and rosuvastatin (ATC-code:C10AA07). Patients who had been prescribed two different types of statins over the observational period of 2 years were excluded ($n = 5{,}361$). The control group (non-statin-treated patients) consisted of patients to whom no statins were dispensed during the observation period. Antidepressant use was measured as the dispensing of at least one antidepressant (ATC code starting with N06A) or in combination with psycholeptics (N06CA), olanzapine (N05AH03), quetiapine (N05AH04), sulpiride (N05AL01), lithium (N05AN01), or benzodiazepine derivatives (N05BA) during the observation period.

Finally, the following groups were defined and compared:

1) Statin-treated patients vs. non-statin-treated patients.
2) Sub-analysis (independent of inpatient care): statin-treated patients on antidepressant medication vs. non-statin-treated patients on antidepressant medication.

Average Daily Doses

Average daily doses for the drugs were calculated from the prescribed dosage, which was converted from defined daily dose to mg and divided by the number of days that were not spent in a hospital. To obtain the individual averages of the daily doses, we extracted the individual drug histories, including information on dates of received prescriptions and the corresponding dosage of the prescribed statin. The average was calculated by dividing the sum of all amounts of the administered drug by the sum of treatment days, minus the days a patient spent in hospital. The hospital days were subtracted on the assumption that the patients were treated with statins during the hospital stay.

In order to ensure precise characterization and interpretation of each substance, we defined groups according to the average daily dose for each statin, resulting in the following categories: >0–10 mg, >10–20 mg, >20–40 mg, >40–60 mg, >60–80 mg.

Ethical Approval

This study has been approved by the ethics commission of the Medical University of Vienna (EK-Nr.: 1020/2020). A detailed statement on ethical approval is provided in the **Supplementary Material**. Written informed consent from the patients was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Statistical Analysis

We calculated odds ratios (ORs) between each case of statin use and diagnosis of MDD in a matched cohort analysis (each statin-treated patient was matched to three members of the control group of the same age and sex). Weighted multiple logistic regression, as described in similar analysis designs (31), was used to investigate this association while controlling for age, sex, dosage, and prescription of other medications (for diabetes and fibrates).

Patients were assigned a categorical variable for each statin comprising the respective average daily dose in mg. We controlled for use of other medications (at least four different quarters during which 20 different glucose-lowering drugs were dispensed, including metformin; three fibrates) by introducing binary dummy variables. Model quality was evaluated using the adjusted R-squared statistic, multi-collinearity via the variance inflation factor (VIF). Stratification was used to control for other diagnoses potentially associated with statin use and MDD and which could in principle act as confounding factors. These robustness tests excluded all patients with a diagnosis of ischaemic heart diseases (any code from the range I20–I25), diseases of the arteries, including arterioles and capillaries (I70–I79), stroke (I63–I64), diabetes (E10–E11), obesity (E66), hypothyroidism (E02–E03), cancer (any code from the range C00–C97), dementia in Alzheimer's disease (specific ICD code for patients with Alzheimer's disease additionally diagnosed with dementia) and Alzheimer's disease (F00, G30), pain (R52), and sleep disorder (G47). Statistical analysis was performed using standard packages of Matlab.

RESULTS

Of a total of 7,481,168 patients, 84,638 (1.13% of the general Austrian population) were diagnosed with MDD during a hospital stay and 888,596 (11.88% of the general Austrian population) were undergoing antidepressant therapy (prescribed independently of a hospital stay). The baseline characteristics of statin-treated patients and their age- and sex-matched control group are described in **Table 1**. In general, statin-treated patients (males and females) were more likely to have been diagnosed with MDD and were more commonly treated with antidepressants when compared to age- and sex-matched non-statin treated patients (OR: 1.54, CI: 1.53–1.56).

Supplementary Table 1 shows the baseline characteristics of the statin-treated patients with diagnosed MDD in comparison to patients with MDD without statin treatment. Depressed patients undergoing statin treatment often received antidepressant and antidiabetic therapy and displayed a higher prevalence rate of CVD, stroke, diseases of the arteries, overweight, obesity and hypothyroidism than those without statin treatment.

Dose-Dependence of Statins on Diagnosis of MDD

In the general population there was an increased risk of diagnosis with depression in statin-treated patients when compared to matched controls (OR: 1.22, CI: 1.20–1.25). Further, there was a potency- and dose-dependent relationship between statins and diagnosis of MDD (see **Table 2**, **Figure 1**, **Supplementary Figures 2, 3** for multiple logistic regression analyses and odds of diagnosis). In comparison to non-statin-treated patients, there was an underrepresentation of diagnosed MDD in patients receiving lovastatin in doses of >0–20 mg (0–10 mg: OR: 0.12, 95% CI: 0.05–0.27; 10–20 mg: OR: 0.62, 95% CI: 0.41–0.95). Similar results were observed for pravastatin >0–20 mg (0–10 mg: OR: 0.36, 95% CI: 0.29–0.44; 10–20 mg: OR: 0.63, CI: 0.56–0.70), simvastatin >0–20 mg (0–10 mg: OR: 0.59, 95% CI: 0.54–0.64; 10–20 mg: OR: 0.81, CI: 0.77–0.86), atorvastatin >0–10 mg (OR: 0.65, 95% CI: 0.59–0.70), rosuvastatin >0–10 mg (OR: 0.68, 95% CI: 0.53–0.85) and fluvastatin >10–60 mg. The results in **Table 2** also demonstrate that the lower risk of diagnosis with MDD in low-dose statin-treated patients decreased with an increase in the potencies of statins. The OR increased with potency, the lowest OR beginning in lovastatin-treated patients. Although there was an underrepresentation of diagnosed MDD in low-dose statin treatment, the higher dosages showed an overrepresentation of MDD. Dosages of >20 mg of simvastatin (20–40 mg: OR: 1.28, 95% CI: 1.21–1.35; 40–60 mg: OR: 2.42, 95% CI: 2.18–2.70; 60–80 mg: OR: 5.27, 95% CI: 4.21–6.60), >10 mg atorvastatin (10–20 mg: OR: 1.19, 95% CI: 1.06–1.33; 20–40 mg: OR: 1.91, 95% CI: 1.60–2.27;40–60 mg: OR: 2.71, 95% CI: 1.98–3.72; 60–80 mg: OR: 3.73, 95% CI: 2.22–6.28) and rosuvastatin >20 mg (20–40 mg: OR: 2.09, 95% CI: 1.31–3.34) were related to an overrepresentation of MDD when compared to controls without statin treatment.

TABLE 1 | Baseline characteristics of the study population, showing group size, age and the absolute and relative frequencies of depression, use of other medications (insulin, oral antidiabetics, antidepressants) and comorbid conditions for males and females in the statin-treated and in the matched control group.

	Statin-treated patients		Non-statin-treated patients	
	Male	**Female**	**Male**	**Female**
N	166,979	170,259	500,937	510,777
Age (mean +/– SD)	64.91 +/- 10.86	68.88 +/– 10.41	64.91 +/– 10.86	68.88 +/– 10.41
Depression (F32–F33)	2,947* (1.76%)	6,774* (3.98%)	6,907 (1.38%)	15,953 (3.12%)
Antidepressants	35,379* (21.19%)	63,534* (37.32%)	73,675 (14.71%)	140,846 (27.57%)
Insulin	10,697* (6.41%)	11,348* (6.67%)	6,281 (1.25%)	6,296 (1.23%)
Oral antidiabetics	32,639* (19.55%)	30,950* (18.18%)	26,605 (5.31%)	25,462 (4.98%)
CVD (I20-I25)	32,611* (19.53%)	20,803* (12.22%)	21,733 (4.34%)	19,631 (3.84%)
Stroke (I63, I64)	4,367* (2.62%)	3,684* (2.16%)	5,826 (1.16%)	5,860 (1.15%)
Diseases of arteries (I70–I79)	10,552* (6.32%)	7,569* (4.45%)	13,227 (2.64%)	10,415 (2.04%)
Overweight and obesity (E66)	7,164* (4.29%)	7,149* (4.20%)	8,384 (1.67%)	11,061 (2.17%)
Hypothyroidism (E02, E03)	1,239* (0.74%)	3,277* (1.92%)	2,551 (0.51%)	6,735 (1.32%)
Alzheimer's disease (F00, G30)	547* (0.33%)	990* (0.58%)	2,537 (0.51%)	4,381 (0.86%)
Pain (R52)	195* (0.12%)	302 (0.18%)	732 (0.15%)	1,003 (0.20%)
Sleep disorders (G47)	2,806* (1.68%)	951* (0.56%)	4,624 (0.92%)	1,878 (0.37%)

*SD, standard deviation; CVD, cardiovascular disease, Asterisks denote statistically significant differences between statin- and non-statin treated patients; **p < 0.01, *p < 0.05.*

TABLE 2 | Dose-dependent relationship between statins and diagnosis of depression.

All	**Lovastatin**	**Fluvastatin**	**Pravastatin**	**Simvastatin**	**Atorvastatin**	**Rosuvastatin**
>0–10 mg	**0.12****	1.00	**0.36****	**0.59****	**0.65****	**0.68****
CI	0.05–0.27	1.00–1.00	0.29–0.44	0.54–0.64	0.59–0.70	0.53–0.85
>10–20 mg	**0.62***	**0.59****	**0.63****	**0.81****	**1.19****	1.20
CI	0.41–0.95	0.43–0.81	0.56–0.70	0.77–0.86	1.06–1.33	0.95–1.53
>20–40 mg	1.29	**0.70****	1.02	**1.28****	**1.91****	**2.09****
CI	0.66–2.51	0.62–0.79	0.91–1.15	1.21–1.35	1.60–2.27	1.31–3.34
>40–60 mg		**0.77****		**2.42****	**2.71****	
CI		0.68–0.86		2.18–2.70	1.98–3.72	
>60–80 mg		1.13		**5.27****	**3.73****	
CI		0.97–1.31		4.21–6.60	2.22–6.28	
Adj. R∧2	0.99	0.98	0.98	0.98	0.99	0.99
Max. VIF	1,61	1.52	1.51	1.93	1.63	1.62

***p < 0.01; *p < 0.05.*
Bold values represent the significant ORs.

Robustness Test for Diseases Commonly Related to MDD

We further conducted a robustness test to estimate the influence of diseases directly related to MDD, in particular ischemic heart disease, diseases of arteries, stroke, diabetes, obesity, hypothyroidism, cancer, dementia in Alzheimer's disease, Alzheimer's disease, pain, and sleep disorders. We thus tested whether the dosage-dependent MDD risk trajectories are independent of the above-mentioned disease groups (see **Figure 2**, **Supplementary Material**: baseline tests and **Supplementary Figures 5–10**). In these robustness tests the results showed that the observed dose dependencies followed the same trend, with an underrepresentation of diagnosed MDD in low-dose and an overrepresentation in high-dose statin-treatment. The dose-dependent relationship could also be observed in a sub-analysis that only included patients hospitalized at least once (see **Supplementary Figure 1**).

Sex-Specific Analysis of the Dose-Dependent Relationship Between Statins and Diagnosis of MDD

In both male and female patients, the results shown in the general population were confirmed, demonstrating that low-dose statin treatment is related to underrepresentation of diagnosed MDD whereas high-dose statin treatment is related to overrepresentation when compared to non-statin-treated patients. Especially for high-dose atorvastatin treatment, we

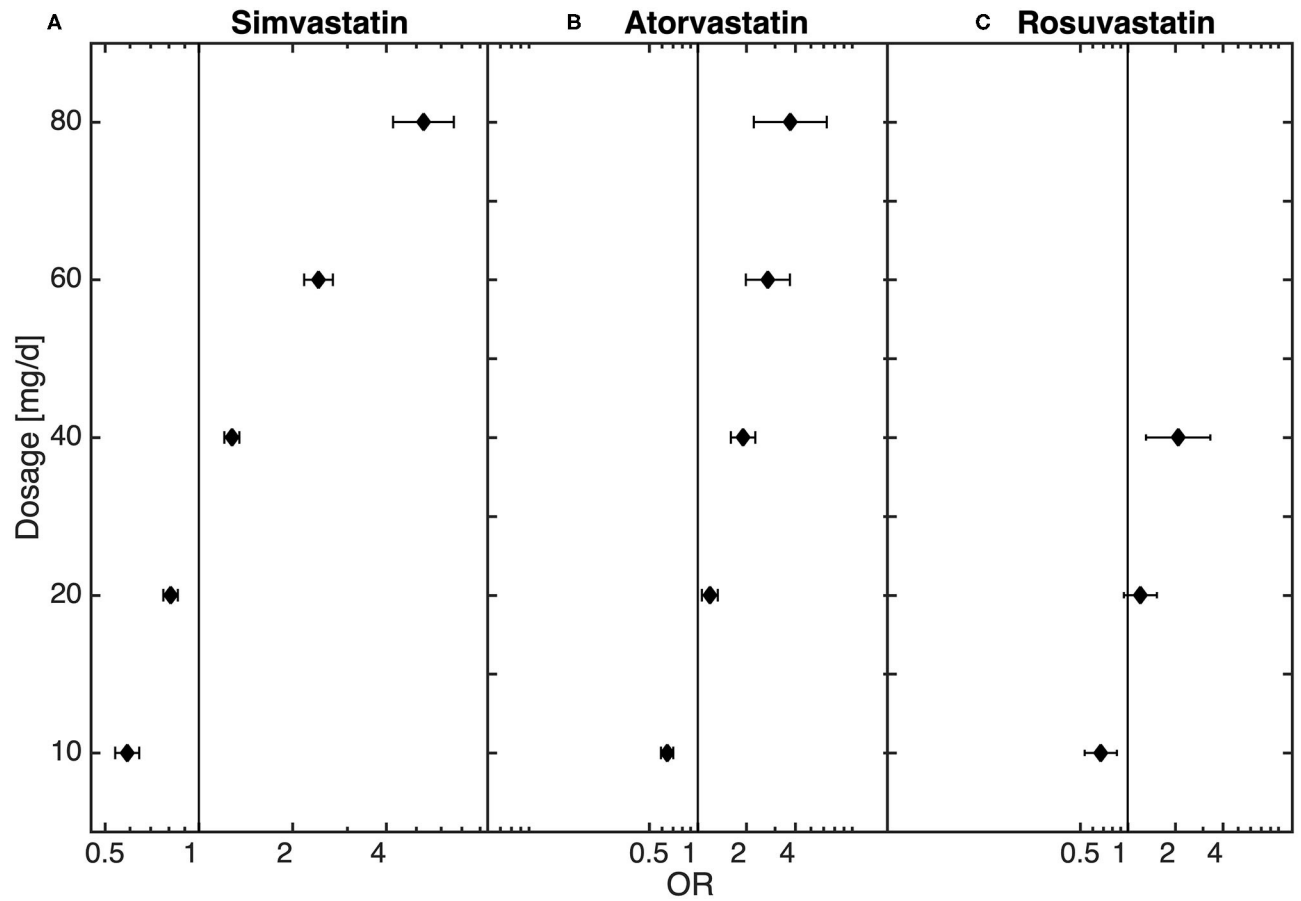

FIGURE 1 | Dose-dependent relationship between **(A)** simvastatin ($n = 214,021$), **(B)** atorvastatin ($n = 37,919$), **(C)** and rosuvastatin ($n = 8,209$) and diagnosis of depression obtained from the logistic regression model.

found that the risk of diagnosis with MDD in females was nearly double than in males. Further details can be found in **Supplementary Tables 2, 3**, **Supplementary Figure 4**.

Sub-Analysis—Dose-Dependent Relationship Between Statin Treatment and Antidepressant Therapy

The final sub-analysis included 888,596 patients receiving antidepressant medication prescribed independently of hospitalization. Statin treatment was recorded for 98,913 of these patients. In the sub-analysis of all patients treated with antidepressants, similar dose-dependent results in statin-treated patients could be observed. Thus, low-dose statin treatment was related to an underrepresentation and high-dose statin treatment to an overrepresentation of antidepressant medication when compared to non-statin-treated patients (**Figure 3**).

DISCUSSION

In the present study, we investigated the relationship between different types and dosages of statins and diagnosis of MDD in comparison to non-statin-treated patients. We were able to demonstrate that there was an increased risk of diagnosed MDD in patients treated with higher doses of statins when compared to non-statin-treated patients. Interestingly, low-dose statin treatment was related to an underrepresentation of MDD when compared to non-statin-treated patients. Our findings are also supported by sex-specific results and displayed no qualitative change after exclusion of patients with diseases closely related to diagnosis of MDD, such as cardiovascular disease or diabetes, and to statin use. The dose-dependent results were also observed in a sub-analysis only including patients taking antidepressant medication prescribed independently of hospitalization.

Given the physiological mechanisms associated with statins, a relationship between statin treatment and mood disorders such as MDD seems plausible. Statins inhibit HMG-CoA reductase, which is the main mechanism for the synthesis of cholesterol, resulting in lower cholesterol levels (32) and consequently subsequent products such as sex hormones (2–5). Close relationships between low sex hormone levels and mood disorders were also suggested (7, 9, 11, 12). We have recently published evidence for a dose-dependent increased risk of diagnosis of osteoporosis in statin-treated patients and

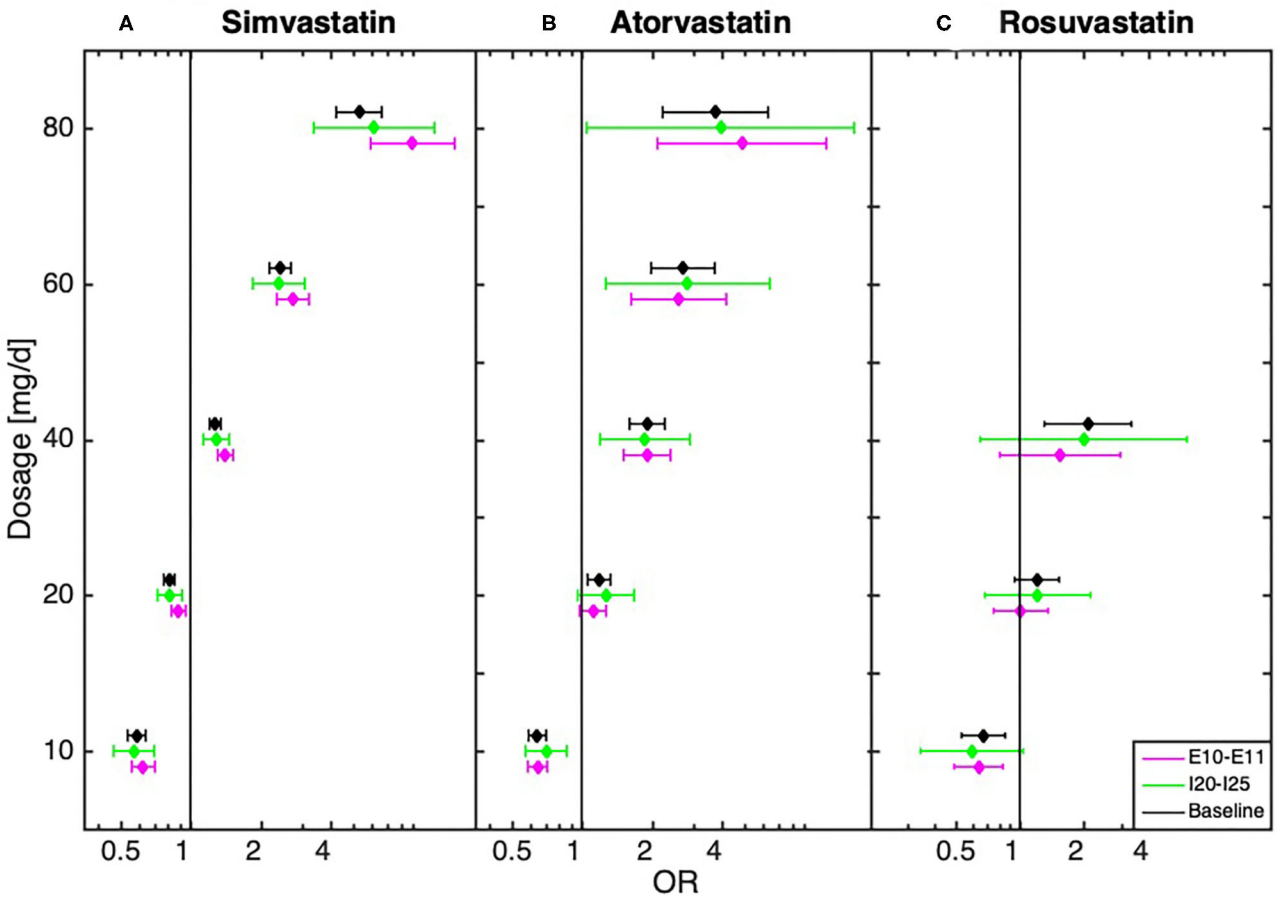

FIGURE 2 | Dose-dependent relationship between **(A)** simvastatin, **(B)** atorvastatin, and **(C)** rosuvastatin and diagnosis of depression for baseline (all statin-treated patients) and after the exclusion of patients with cardiovascular disease (I20–I25, $n = 53{,}414$) and ($n = 71{,}722$)/or diabetes (E10–E11, $n = 32{,}815$) obtained from the logistic regression model.

hypothesized that specifically higher dosages could inhibit the synthesis of sex hormones on a clinically relevant level and therefore advance bone resorption (33). By showing that there is also a dose-dependent relationship with diagnosed MDD, here we demonstrate for the first time that it is important to consider the different types of statins and their dosages when investigating the relationship between statin treatment and diagnosis of MDD.

Concerns about potential central nervous side-effects of statins have been raised for almost 30 years. A series of pioneering work made a case for increased depression rates as well as non-cardiovascular mortality due to violent incidents and suicide in patients with low cholesterol and thus in statin users (34–39). Interestingly, these early positive associations were rarely replicated (17), as more recent large clinical and register-based studies have generally implied no increased risk of developing MDD in statin users (22). On the contrary, protective effects have been reported and were related to neuroinflammation as a potential key mechanism of depression (40–42). Nevertheless, overall the mechanisms of putative association in either direction are insufficiently understood. Most importantly, the majority of studies have neglected different potencies and dosages of statins despite recent advances linking statins with high lipophilia and hence permeability of the blood-brain barrier but not establishing a connection between hydrophilic statins and MDD (43). Hence it is not clear whether under high-dose statin therapy a stronger downregulation of sex hormones, which are closely related to MDD, could possibly overrule the positive anti-inflammatory effect of statins on MDD.

Data regarding the dose-dependent risk of MDD in statin-treated patients are particularly sparse. For instance, in placebo-controlled clinical trials it has been shown that both low-dose lovastatin treatment (30 mg) (44) and low-dose simvastatin treatment (20 mg) (45, 46) resulted in significant relief of depressive symptoms. There are also other prospective studies demonstrating that low-dose statin treatment, such as 20 mg of atorvastatin (46, 47), could have positive effects on symptoms of depression. These results indicate that low-dose statin treatment could indeed be effective in the treatment of depression and are consequently in accordance with our results, as we found an underrepresentation of MDD in patients treated with lower doses of lovastatin (0–20 mg), pravastatin (0–20 mg), simvastatin (0–20 mg), atorvastatin (0–10 mg), and rosuvastatin (0–10 mg).

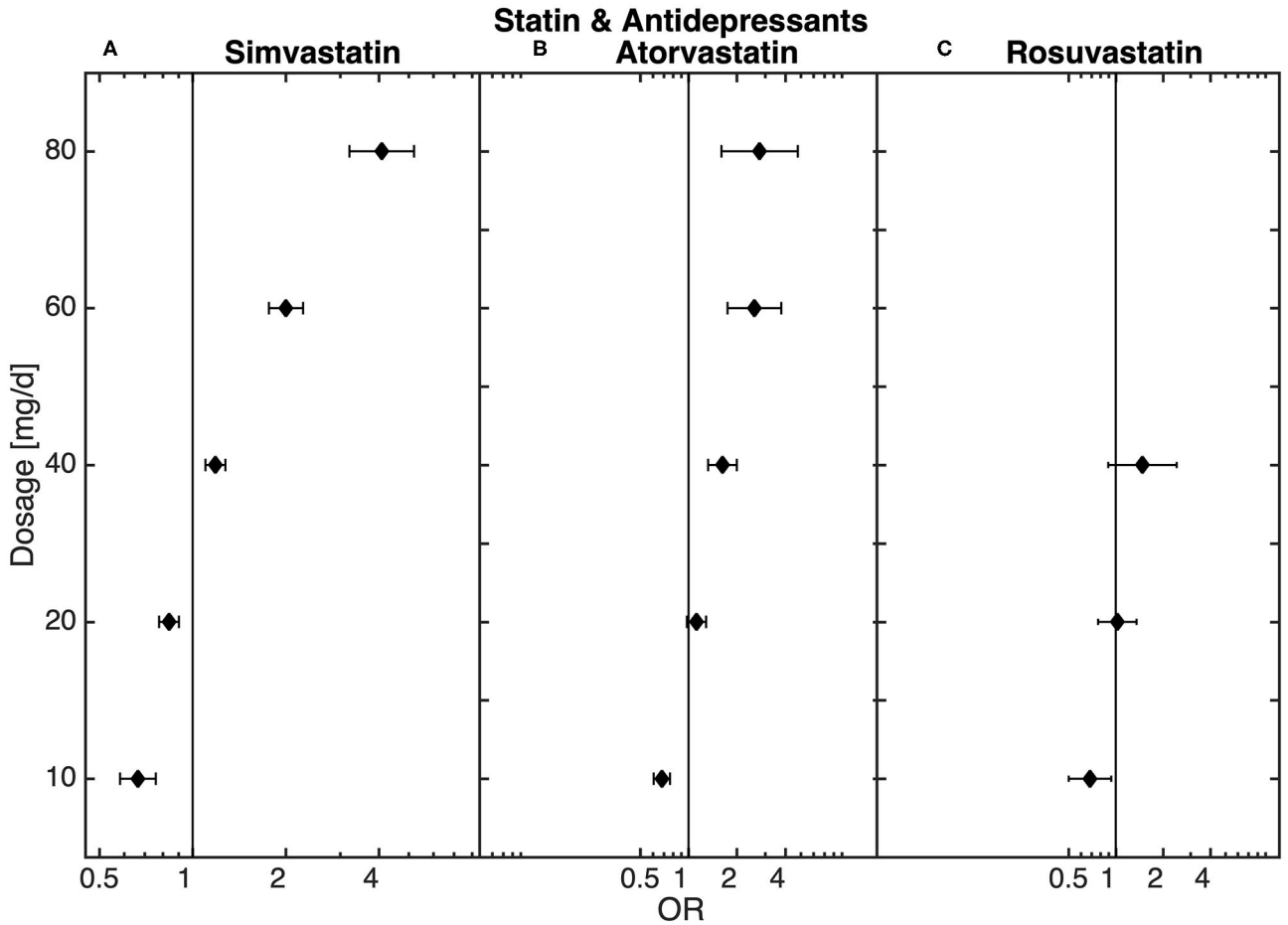

FIGURE 3 | Dose-dependent relationship between **(A)** simvastatin, **(B)** atorvastatin, and **(C)** rosuvastatin and antidepressant medication ($n = 98{,}913$) obtained from the logistic regression model.

There is evidence that statins can have positive effects on depression via an anti-inflammatory effect, modulation of cytokines and reduction of oxidative stress. Improved quality of life due to improved health consciousness and treatment compliance and reduced cardiovascular risk has also been linked to a potential antidepressant effect of statins (48). Nevertheless, especially the relationship to higher dosages has yet to be investigated sufficiently. That it is also important to investigate the different types of statins with their different potencies has been demonstrated by a Swedish national cohort study showing that simvastatin had a protective effect on depression, whereas atorvastatin treatment increased the risk. However, there was no detailed investigation of different dosages (25).

In the present study, higher dosages were related to an overrepresentation of MDD in statin-treated patients. These results could be confirmed by a sex-specific analysis and remain unchanged after exclusion of patients with diseases closely related to MDD. Further, similar results were observed in a sub-analysis investigating all patients taking antidepressant medication prescribed independently of hospitalization. Thus, dosage and potency may be the deciding factors for protective or risk-increasing effects. Whether a possible downregulation of hormones directly related to MDD under high-dose statin treatment could overwhelm the described positive anti-inflammatory effects of statins on MDD should be investigated in larger prospective clinical trials. The overrepresentation of diagnosed MDD in high-dose statin-treated patients is of special interest with regard to the synthesis and processing of cholesterol to essential hormones such as steroid hormones or Vitamin D. A study by Chan et al. investigated the effect of high-dose 80 mg simvastatin therapy on mood in a cohort of 140 patients with secondary progressive multiple sclerosis by conducting a 24 month, double-blind controlled trial. They showed similar results to those of our study, namely that high-dose statin treatment was related to increased severity of depressive symptoms, as measured using the Hamilton Depression Rating Scale (HAM-D) (49). Hence the fact that cholesterol is required for the serotonin 1A receptor to function (18–20) also has to be considered, since down-regulation of this receptor is closely related to mood disorders (21).

Possible interactions with the metabolization of statins should not be discounted either, as in our study females were at over double the risk of diagnosis with MDD than males when receiving high-dose atorvastatin treatment. One thus has to

consider that atorvastatin is metabolized by cytochrome P450 3A4 (CYP3A4), which is also involved in the metabolism of estrogens.

Our study has both limitations and strengths. First, register-based studies are limited by the fact that there is no opportunity to precisely characterize the diagnosis of the diseases using clinical data, since we only have access to ICD codes. In the present study, the number of patients diagnosed with depression was in relation to the actual literary lower. However, a strength is that antidepressant medication was also recorded independently of hospital stays and this number reflects the actual literary and might compensate underreporting in the second group. A further limitation of the present study is that we do not have information on the duration of statin treatment and that we cannot infer causal effects using the cross-sectional study design. Additionally, it is not possible to screen the data for potential associations between different time periods of statin treatment and the relationship to major depressive disorders and/or antidepressant medication. Hence especially high-dose statin-treated patients often had a history of cardiovascular events and a higher occurrence of cardiovascular risk markers and thus MDD could therefore be a consequence of CVD (50, 51). In our dataset from 2006 to 2007 we had no access to cholesterol levels and could therefore only hypothesize that there could be a dose dependent relationship between statins and the upcoming synthesis of vital hormones (e.g., testosterone and estrogen), which are directly related to MDD. Additionally, we have no information on marital and socioeconomic status, which is also related to MDD. Also, we have no information about common side effects of statins, such as muscle symptoms or reduced exercise tolerance, and it is known that these symptoms may induce mood disorders. Further, it was not possible to provide detailed analysis of treatment adherence. In comparison to controls, statin-treated patients are characterized in the present study by a higher rate of comorbidities and it is a well-known fact that diseases such as diabetes mellitus or CHD, for instance, are closely related to the development of MDD. However, in our robustness tests, there was no qualitative change in the results after the exclusion of patients with such diagnoses.

CONCLUSION

In conclusion, our results demonstrate that there exists a dose-dependent relationship between statins and diagnosis of MDD, substantiating both underrepresentation of MDD in low-dose statin treatment and increased risk of diagnosis with MDD in high-dose treatment. Considering the widespread use of statins primarily for disease prevention and increasingly stricter recommendations for tolerated cholesterol levels, these findings may be highly relevant for clinical routine across a broad spectrum of medical disciplines. This is an important and interesting approach for precision medicine in particular. Nevertheless, keeping in mind the limitations of register-based studies, prospective and longitudinal trials are urgently needed to validate our findings and further elucidate the mechanisms involved.

DATA AVAILABILITY STATEMENT

The data is not available to access because this is a consolidated research database that is only accessible for selected partners under a strict data protection policy.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical University of Vienna. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

ML, CM, AK, MK, ST, PK, and AK-W: study design. ML, CM, and PK: data analysis. ML and CM: manuscript writing. AK-W: is the guarantor of this work. All authors read, reviewed, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.608083/full#supplementary-material

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CORRECTION
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Corrigendum: Major Depressive Disorder (MDD) and Antidepressant Medication Are Overrepresented in High-Dose Statin Treatment

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A Corrigendum on

Major Depressive Disorder (MDD) and Antidepressant Medication Are Overrepresented in High-Dose Statin Treatment

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In the original article, the authors' name and surname were swapped. The correct author list appears in this article. This has been corrected in all relevant sections. The citation was corrected to "Leutner M, Matzhold C, Kautzky A, Kaleta M, Thurner S, Klimek P and Kautzky-Willer A (2021) Major Depressive Disorder (MDD) and Antidepressant Medication Are Overrepresented in High-Dose Statin Treatment. *Front. Med.* 8:608083. doi: 10.3389/fmed.2021.608083."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Sex Differences in the Incidence and Risk Factors of Myocardial Injury in COVID-19 Patients: A Retrospective Cohort Study

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Male novel coronavirus disease (COVID-19) patients tend to have poorer clinical outcomes than female patients, while the myocardial injury is strongly associated with COVID-19-related adverse events. Owing to a lack of corresponding data, we aimed to investigate the sex differences in the incidence of myocardial injury in COVID-19 patients and to identify the potential underlying mechanisms, which may partly account for the sex bias in the incidence of adverse events. This retrospective study included 1,157 COVID-19 patients who were hospitalized in Huoshenshan Hospital from 12 March 2020 to 11 April 2020. Data on the patients' demographic characteristics, initial symptoms, comorbidities and laboratory tests were collected. Totally, 571 (49.4%) female and 586 (50.6%) male COVID-19 patients were enrolled. The incidence of myocardial injury was higher among men than women (9.2 vs. 4.9%, $p = 0.004$). In the logistic regression analysis, age, and chronic kidney disease were associated with myocardial injury in both sexes. However, hypertension [odds ratio (OR) = 2.25, 95% confidence interval (CI) 1.20–4.22], coronary artery disease ($OR = 2.46$, 95% CI 1.14–5.34), leucocyte counts ($OR = 3.13$, 95% CI 1.24–7.86), hs-CRP ($OR = 4.45$, 95% CI 1.33–14.83), and D-dimer [$OR = 3.93$ (1.27–12.19), 95% CI 1.27–12.19] were independent risk factors only in the men. The correlations of hs-CRP and D-dimer with hs-cTnI and BNP were stronger in the men. The incidence of myocardial injury in COVID-19 patients is sex-dependent, predominantly in association with a greater degree of inflammation and coagulation disorders in men. Our findings can be used to improve the quality of clinical management in such settings.

Keywords: COVID-19, sex differences, myocardial injury, risk-factors, inflammation, coagulation disorder

INTRODUCTION

As of November 2020, the novel 2019 coronavirus disease (COVID-19) has led to more than 55 million confirmed cases worldwide, including nearly 1.5 million deaths (World Health Organization, 2020b). The mortality associated with the disease ranges from 5.8 to 11.7% (Du et al., 2020; Mehra et al., 2020; Shi et al., 2020b). Studies focusing on the epidemiological and

clinical characteristics of COVID-19 have shown that, in addition to old age and comorbidities, sex differences are also associated with disease deterioration and mortality (Chen et al., 2020; Yang et al., 2020), with male patients showing significantly higher mortality values (Epidemiology Working Group for NCIP Epidemic Response, 2020). In Spain, the mortality among male COVID-19 patients is twice as high as that among their female counterparts (Pastor-Barriuso et al., 2020). Another observational, longitudinal study on 10-year mortality, enrolled 1,284 subjects without COVID-19, demonstrated that males tended to have a lower prevalence of frailty and comorbidities, receive fewer drugs, but have higher mortality than females (Corbi et al., 2019). However, there remains a lack of clarity on the underlying reasons for the sex differences in the incidence of fatal outcomes in such settings. In COVID-19, mortality is strongly associated with the incidence of myocardial injury (7.2 to –27.8%) (Guo et al., 2020; Huang et al., 2020; Wang et al., 2020). Moreover, myocardial injury development may result in the deterioration of other COVID-19-related outcomes [e.g., acute respiratory distress syndrome (ARDS), intensive care unit (ICU) admission and ventilator therapy] (Lala et al., 2020; Lombardi et al., 2020; Shi et al., 2020b). Therefore, the sex differences in the incidence of COVID-19-related myocardial injury may partly account for the sex bias in the incidence of adverse events. While some studies showed that the incidence of myocardial injury is higher among men than women (Guo et al., 2020), others did not observe significant sex-related differences (Shi et al., 2020b). Thus, whether the incidence of COVID-19-related myocardial injury is sex-dependent remains controversial.

Therefore, we aimed to retrospectively compare the epidemiological characteristics, laboratory test results, and risk factors associated with myocardial injury between female and male COVID-19 patients to identify sex differences in the incidence of myocardial injury as well as the underlying potential mechanisms so as to facilitate optimal clinical management.

MATERIALS AND METHODS

Study Design and Participants

A total of 1,201 patients who were hospitalized at Huoshenshan Hospital (Wuhan, China) from 12 March 2020 to 11 April 2020 and diagnosed with laboratory-confirmed COVID-19 according to World Health Organization guidelines (World Health Organization, 2020a) were enrolled in this single-center, retrospective cohort study. Patients (1) aged under 18 years, (2) without laboratory test data, or (3) without high-sensitivity cardiac troponin I (hs-cTnI) test results were excluded (Shi et al., 2020b). Real-time reverse transcriptase-polymerase chain reaction performed using throat swab specimen was employed for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection detection.

Our study protocol was approved by the Human Ethics Committee, Huoshenshan Hospital (No. HSSLL023). The study conformed to the ethical guidelines of the Declaration of Helsinki. Given the limited medical resources and the need to treat a large volume of patients in the urgently constructed hospital in a short time, it was a huge challenge to gather the informed consent form for every hospitalized patient. Oral informed consent was approved by the ethics commission of the hospital for patients with COVID-19 (Shi et al., 2020a).

Data Collection

Data on the patients' demographics, initial symptoms, comorbidities, and laboratory tests (routine blood test, renal and liver function, coagulation profile, cardiac biomarkers, inflammatory biomarkers) were obtained from standardized clinical electronic medical records. Laboratory tests were completed within 1 day after admission. All data were independently verified and entered into the computer database by two experienced physicians.

Definition

Age was classified as $\leq$65 years and >65 years (Du et al., 2020). Initial symptoms were defined as the first symptoms that appeared in the early infection stages. Comorbidities were diagnosed using the International Classification of Disease 10 codes before SARS-CoV-2 infection. Laboratory tests were classified as normal or abnormal based on Huoshenshan Hospital criteria (**Table 2**). ARDS was defined according to the Berlin Definition (Ranieri et al., 2012). Myocardial injury was confirmed if the hs-cTnI level was higher than the 99th percentile upper reference limit (Thygesen et al., 2018). According to the guidelines for diagnosis and management of COVID-19 (5th version, in Chinese) released by the National Health Commission of China, the severe and critically ill cases was defined when meeting any of the follows: respiratory rate $\geq$30 times/min; pulse oxygen saturation $\leq$93% at rest; arterial oxygen partial pressure/fraction of inspired oxygen $\leq$300 mmHg; respiratory failure requiring mechanical ventilation; or respiratory failure combined with other organ failure requiring ICU treatment (National Health Commission of China, 2020).

Statistical Analysis

Continuous variables were represented as medians (25th–75th percentile). Independent sample *t*-tests or Mann-Whitney *U* tests were used for the comparison of continuous variables between the groups according to the distribution. Categorical data were exhibited as counts and percentages and further analyzed by the Chi-squared test or Fisher's exact test when appropriate.

Logistic regression analyses were applied to determine the independent risk factors for myocardial injury. Variables with $p < 0.1$ in the univariable analysis or those that were considered clinically relevant were entered into the multivariable models. Linear regression was applied for the assessment of the associations between cardiac biomarkers and potential risk factors. The standardized regression coefficient (R) was used to describe the association. Forest plots were applied to display the results of the multiple logistics regression analysis. A two-tailed $P < 0.05$ was considered statistically significant.

Statistical analyses were performed with SPSS 26.0 software (IBM Corp., Armonk, NY, United States). Data visualization was generated by Prism 7.0 (GraphPad Software Inc., San Diego, CA, United States).

RESULTS

Baseline Characteristics in the Male and Female Patients With and Without Myocardial Injury

A flow chart of the patient recruitment process is presented in **Figure 1A**. Briefly, a total of 1,201 COVID-19 patients were admitted to Huoshenshan Hospital from 12 March 2020 to 11 April 2020. After the exclusion of two patients aged under 18 years, 27 without laboratory test data and 15 without hs-cTnI test data, 1,157 patients were included in our final analysis, comprising 571 (49.4%) women and 586 (50.6%) men. Among the 1,157 COVID-19 patients included in our study, a significantly higher incidence of myocardial injury was observed in men than women (9.2 vs. 4.9%, $p = 0.004$) (**Figure 1B**).

The baseline characteristics of the patients with and without myocardial injury are summarized in **Table 1**. In both sexes, the presence of myocardial injury was associated with older age [women: 67.5 (57.5–75.75) vs. 61 (53–68) years, $p = 0.004$, men: 72 (63.75–78) vs. 61 (51–69) years, $p < 0.001$] and higher frequencies of hypertension [women: 15 (53.6%) vs. 193 (35.3%), $p = 0.053$; men: 35 (64.8%) vs. 177 (33.3%), $p < 0.001$] and chronic kidney disease (CKD) [women: 3 (10.7%) vs. 9 (1.7%), $p = 0.017$; men: 5 (9.3%) vs. 9 (1.7%), $p = 0.003$)].

However, in the men alone, the frequencies of coronary artery disease (CAD) [14 (25.9%) vs. 34 (6.4%), $p < 0.001$] and cerebrovascular disease [8 (14.8%) vs. 24 (4.5%), $p = 0.004$] were higher in those with myocardial injury than in those without it. Moreover, compared to their counterparts without this injury, the male myocardial injury patients had a higher incidence of nausea/vomiting [4 (7.4%) vs. 10 (1.9%), $p = 0.039$] as an initial symptom but a lower incidence of fever [29 (53.7%) vs. 391 (73.5%), $p = 0.002$]. Both in females and males, patients with myocardial injury exhibited more severe and critically ill cases and had poor clinical outcomes, such as respiratory failure, ARDS, ICU admission, and death (all $p < 0.001$).

Laboratory Findings at Admission in Male and Female Patients With and Without Myocardial Injury

Both the male and female myocardial injury patients had higher levels of creatine kinase-MB, lactic dehydrogenase, α-hydroxybutyrate dehydrogenase, hs-cTnI, brain natriuretic peptide (BNP), myoglobin, leucocytes, high-sensitive C-reactive protein (hs-CRP), urea nitrogen, aspartate aminotransferase, prothrombin time and D-dimer but a lower lymphocyte percentage and monocyte percentage (all $p < 0.05$) (**Supplementary Table S1**).

Risk Factors for Myocardial Injury in COVID-19 Patients According to Sex

In the univariable regression analysis, age (>65 years), history of hypertension, and CKD cerebrovascular disease were risk factors for the incidence of myocardial injury in both sexes. However, in the men alone, CAD and cerebrovascular disease were associated with the incidence of myocardial injury. In the multivariable logistic regression analysis conducted among the female patients, age (>65 years) [odds ratio (OR) = 3.76, 95% confidence interval (CI) 1.61–8.77, $p = 0.002$], history of CKD ($OR = 4.28$, 95% CI 1.02–18.06, $p = 0.048$ were independent risk factors for the incidence of myocardial injury. Among the male patients, age (>65 years) ($OR = 4.02$, 95% CI 2.05–7.90, $p < 0.001$), history of hypertension ($OR = 2.25$ 95% CI (1.20–4.22, $p = 0.012$), CAD ($OR = 2.46$, 95% CI 1.14–5.34, $p = 0.022$) and CKD ($OR = 4.76$, 95% CI 1.38–16.40, $p = 0.013$) were independently associated with the incidence of myocardial injury (**Supplementary Table S2** and **Figure 2**). In terms of laboratory variables, after multivariable adjustment for age and the above-mentioned comorbidities, the leucocyte count ($OR = 3.13$, 95% CI 1.24–7.86, $p = 0.016$), the levels of hs-CRP ($OR = 4.45$, 95% CI 1.33–14.83, $p < 0.001$) and D-dimer ($OR = 3.93$, 95% CI 1.27–12.19, $p = 0.018$) were determined as being independently related to myocardial injury only in the male patients (**Table 2**).

Correlations Between hs-CRP, D-Dimer and Biomarkers of Myocardial Injury in COVID-19 Patients According to Sex

Compared to the female myocardial injury patients, the male patients had a remarkable increase in levels of BNP [167.71 (38.47–611.47) vs. 59.13 (6.25–305.97) pg/mL, $p < 0.05$], hs-CRP [51.07 (14.27–115.39) vs. 10.57 (1.13–79.22) mg/L, $p < 0.05$] and D-dimer [4.29 (1.20–9.05) vs. 1.16 (0.54–3.69) mg/L, $p < 0.01$]. However, hs-cTnI level did not manifest the significant sex difference in myocardial injury patients ($p > 0.05$) (**Figure 3**).

In the linear regression analysis, the levels of hs-CRP were positively correlated with those of hs-cTnI in the men ($R = 0.202$, $p < 0.001$) but not women. Furthermore, the levels of D-dimer were correlated with the hs-cTnI values in both the men ($R = 0.268$, $p < 0.001$) and women ($R = 0.157$, $p < 0.001$). The levels of hs-CRP (men: $R = 0.354$, $p < 0.001$; women: $R = 0.273$, $p < 0.001$) and D-dimer (men: $R = 0.501$, $p < 0.001$; women: $R = 0.153$, $p = 0.003$) were correlated with those of BNP in both sexes. However, the correlations were stronger in the men than women (**Figure 3**).

DISCUSSION

In the present study, we found that the incidence of myocardial injury was higher among male COVID-19 patients than their female counterparts. The multivariate logistic regression analyses showed that old age and CKD were independently associated with the presence of myocardial injury in both sexes. However, history of hypertension and CAD, the elevated hs-CRP and D-dimer levels were independent risk factors only in the men. Additionally, while correlations of hs-CRP and D-dimer with cardiac biomarkers were observed in both sexes, they were more pronounced in men. Our findings suggest the presence of sex differences in the incidence and risk factors of myocardial injury in COVID-19 patients.

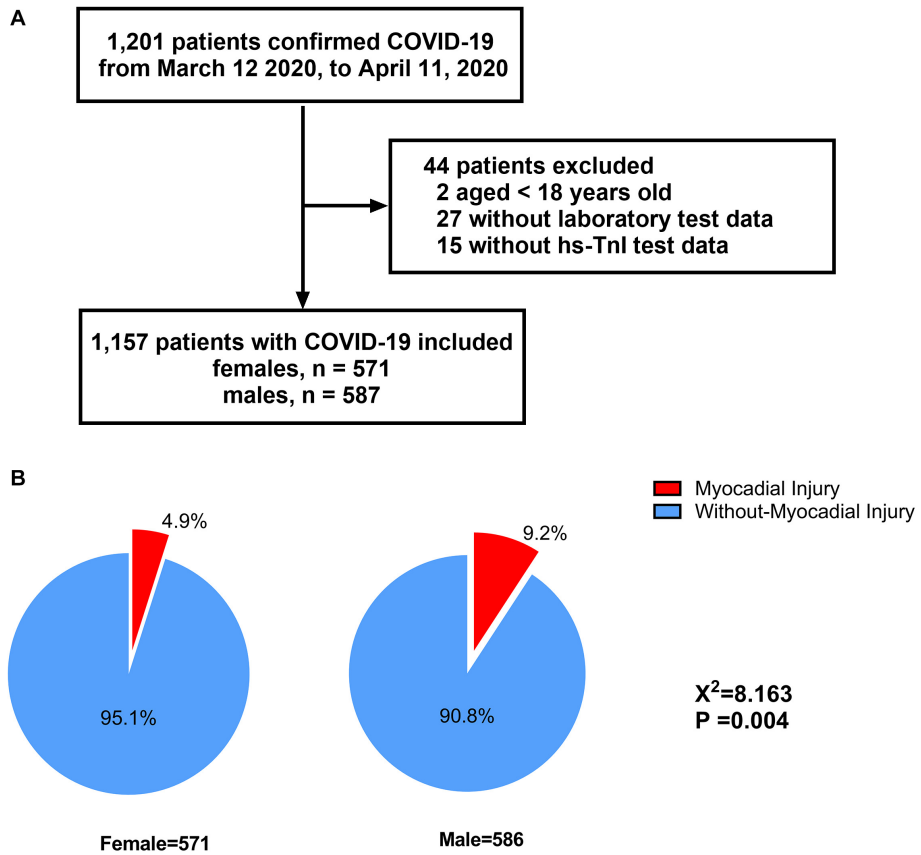

FIGURE 1 | Flowchart of patients recruitment **(A)** and distribution of myocardial injury with regard to different sex inpatients with COVID-19 **(B)**.

Sex Differences in the Incidence of Myocardial Injury and Impacts on Cardiac Function

As illustrated above, the sex bias in the incidence of myocardial injury in COVID-19 patients is controversial. Although some studies reported the absence of significant sex differences in the incidence of myocardial injury (Lombardi et al., 2020; Shi et al., 2020b), our large-scale study suggested that men are likelier to develop it. Interestingly, the absolute value of hs-TnI did not manifest the sex disparities in myocardial injury patients, suggested that no sex difference existed in the severity of myocardial injury even if males were more prone to it. Similarly, a study of 2,736 COVID-19-positive individuals in New York City reported that no significant sex differences when the severity of myocardial injury was stratified by troponin I degrees (Lala et al., 2020).

In the present study, those with myocardial injury showed a marked increase in their BNP levels in association with serious cardiac function impairment (Troughton et al., 2014). Previous studies that focused on the cardiovascular implications of COVID-19 found that the mean concentration of BNP was much higher in those who died, highlighting the prognostic significance of this parameter (Guo et al., 2020). Accordingly, it can be hypothesized that patients with a higher risk of severe COVID-19 progression and outcome due to myocardial injury and worse cardiac function may include a disproportionate number of males.

Sex Differences in Risk Factors for Myocardial Injury

Consistent with previous studies (McCarthy et al., 2019; Shi et al., 2020a), the myocardial injury patients in our study tended to be older and have a larger number of pre-existing illnesses (hypertension and CKD) suggesting that these comorbidities accelerate the development of myocardial injury. Furthermore, male patients were older and had higher incidences of CAD and cerebrovascular disease. In line with our findings, Guo et al. reported that the proportions of men were higher than those of women in elderly populations and populations with coronary heart disease (Guo et al., 2020).

Generally, elderly males suffer from a more serious reduced in total numbers of immune cells and inverted CD4/CD8 T-cell ratio as compared with the female (Perrotta et al., 2020), resulting in the impaired immunologic surveillance and immune clearance function in aging males. Moreover, Svartengren et al. (2005) demonstrated the clearance function of inhaled particles in small airway areas decreased with age. In addition, upper airway size as well decreases in

TABLE 1 | Comparison of demographics, initial symptoms, and comorbidities between myocardial injury and without-myocardial injury in female and male COVID-19 patients.

	Female			Male		
	Myocardial injury ($n = 28$)	**Without-myocardial injury ($n = 543$)**	***p*-value**	**Myocardial injury ($n = 54$)**	**Without-myocardial injury ($n = 532$)**	***p*-value**
Demographics						
Age, years	67.5 (57.5–75.75)	61 (53–68)	0.004	72 (63.75–78)	61 (51–69)	<0.001
Current smoker, n (%)	0 (0)	1 (0.2)	1.000	5 (9.3)	67 (12.6)$^{\#\#}$	0.477
Initial symptoms						
Fever (≧37.3°C), n (%)	18 (64.3)	367 (67.6)	0.716	29 (53.7)	391 (73.5)$^{\#}$	0.002
Cough, n (%)	18 (64.3)	382 (70.3)	0.494	32 (59.3)	337 (63.3)$^{\#}$	0.553
Sputum, n (%)	2 (7.1)	67 (12.3)	0.599	10 (18.5)	69 (13.0)	0.255
Short of breath, n (%)	14 (50.0)	241 (44.4)	0.560	26 (48.1)	239 (44.9)	0.650
Fatigue, n (%)	11 (39.3)	196 (36.1)	0.732	13 (24.1)	187 (35.2)	0.102
Nausea/vomiting, n (%)	1 (3.6)	18 (3.3)	1.000	4 (7.4)	10 (1.9)	0.039
Stuffy/runny noses, n (%)	0 (0)	4 (0.7)	1.000	0 (0)	2 (0.4)	1.000
Throat discomfort, n (%)	0 (0)	27 (5.0)	0.452	1 (1.9)	10 (1.9)$^{\#\#}$	1.000
Comorbidities						
Hypertension, n (%)	15 (53.6)	193 (35.5)	0.053	35 (64.8)	177 (33.3)	< 0.001
Diabetes, n (%)	7 (25.0)	81 (14.9)	0.241	13 (24.1)	91 (17.1)	0.202
Arrhythmia, n (%)	1 (3.6)	24 (4.4)	1.000	4 (7.4)	20 (3.8)	0.353
Malignant neoplasms, n (%)	1 (3.6)	12 (2.2)	0.484	3 (5.6)	14 (2.6)	0.427
CAD, n (%)	2 (7.1)	37 (6.8)	1.000	14 (25.9)*	34 (6.4)	<0.001
COPD, n (%)	1 (3.6)	15 (2.8)	0.560	4 (7.5)	29 (5.5)$^{\#}$	0.758
CLD, n (%)	1 (3.6)	9 (1.7)	0.398	3 (5.6)	21 (3.9)$^{\#}$	0.835
CKD, n (%)	3 (10.7)	9 (1.7)	0.017	5 (9.3)	9 (1.7)	0.003
Anemia, n (%)	1 (3.6)	10 (1.8)	0.428	3 (5.6)	8 (1.5)	0.118
Cerebrovascular disease, n (%)	1 (3.6)	18 (3.3)	1.000	8 (14.8)	24 (4.5)	0.004
Clinical outcomes						
Respiratory failure, n (%)	10 (35.7)	20 (3.7)	<0.001	28 (51.9)	26 (4.9)	<0.001
ARDS, n (%)	11 (39.3)	15 (2.8)	<0.001	25 (46.3)	23 (4.3)	<0.001
ICU admission, n (%)	8 (28.6)	18 (3.3)	<0.001	25 (46.3)	25 (4.7)	<0.001
Death, n (%)	8 (28.6)	6 (1.1)	<0.001	19 (35.2)	9 (1.7)	<0.001
Disease severity						
Mild, n (%)	10 (35.7)	398 (73.3)	<0.001	20 (37.0)	362 (68.0)	<0.001
Severe and critically ill, n (%)	18 (64.3)	145 (26.7)		34 (63.0)	170 (32.0)	

*Continuous variables with non-normal distribution were represented as medians (25th–75th percentile) and categorical data were represented as count and percentage. COVID-19, novel 2019 coronavirus disease; COPD, chronic obstructive pulmonary disease; CAD, coronary artery disease; CLD, chronic liver disease; CKD, chronic kidney disease; ARDS, acute respiratory distress syndrome; ICU, intensive care unit admission. *$p < 0.05$, compared to female patients with myocardial injury. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared to female patients without myocardial injury.*

an age-related manner, which is more pronounced in males (Martin et al., 1997).

These viewpoints partly account for the sex differences in COVID-19 infection rate and are indispensable to the further interpretation of the higher myocardial incidence in aging males compared to aging females (Perrotta et al., 2020).

Sex Differences in the Mechanism of Myocardial Injury

To characterize cardiac structural and functional abnormalities of COVID-19 patients, echocardiographic and electrocardiographic data have been analyzed by several researchers. Giustino et al. (2020) reported that patients with myocardial injury more suffered from left ventricle dysfunction, regional wall motion abnormalities, right ventricle dysfunction, and pericardial effusions. Additionally, recently researches assessed by speckle-tracking echocardiography supported that worsening left ventricle and right ventricle function, reflected by reduced global and regional strain, were more observed in patients with severe COVID-19 infection and more associated with poorer grade and clinical deterioration (Lassen et al., 2020; Rothschild et al., 2020). Meanwhile, 12-lead electrocardiogram identified that two different patterns of ST-segment changes, including global biventricular dysfunction related diffuse ST-segment changes and regional wall motion abnormalities associated regional ST-segment changes (Giustino et al., 2020). It was worth noting that the sex differences in cardiac structural and functional characteristic changes were not been reported.

TABLE 2 | Association between laboratory findings and myocardial injury in female and male COVID-19 patients.

Variables		Female				Male			
		Univariable OR (95%CI)	*p*-value	Multivariable OR (95%CI)	*p*-value*	Univariable OR (95%CI)	*p*-value	Multivariable OR (95%CI)	*p*-value*
Leucocytes, $\times10^9$/L	4–10	1 (Ref)				1 (Ref)			
	<4 or >10	3.56 (1.61–7.86)	0.002	1.97 (0.59–6.58)	0.271	2.70 (1.48–4.94)	0.001	3.13 (1.24–7.86)	0.016
Neutrophil percentage, %	40–75	1(Ref)				1 (Ref)			
	<40 or >75	6.36 (2.80–14.41)	<0.001	2.90 (0.83–10.14)	0.095	9.07 (4.98–16.51)	<0.001	1.86 (0.71–4.85)	0.205
Hemoglobin, g/L	≥115	1 (Ref)				1 (Ref)			
	<115	1.87 (0.87–4.00)	0.109	0.34 (0.11–1.10)	0.073	3.47 (1.95–6.16)	<0.001	0.94 (0.38–2.35)	0.900
Platelets, $\times10^9$/L	100–300	1 (Ref)				1 (Ref)			
	<100 or >300	1.81 (0.80–4.11)	0.156	0.83 (0.26–2.69)	0.761	2.65 (1.47–4.77)	0.001	1.77 (0.67–4.65)	0.247
hs–CRP, mg/L	≤4	1 (Ref)				1 (Ref)			
	>4	5.53 (2.39–12.82)	<0.001	2.21 (0.67–7.32)	0.193	16.40 (6.42–41.92)	<0.001	4.45 (1.33–14.83)	0.015
ALT, IU/L	≤50	1 (Ref)				1 (Ref)			
	>50	0.78 (0.18–3.4)	0.745	1.02 (0.17–5.96)	0.985	1.30 (0.67–2.51)	0.437	1.07 (0.40–2.87)	0.900
TBil, μmol/L	≤26	1 (Ref)				1 (Ref)			
	>26	5.30 (1.07–26.27)	0.041	7.38 (0.98–55.76)	0.053	3.84 (0.99–14.92)	0.052	3.88 (0.37–40.51)	0.257
BUN, mmol/L	≤9.5	1 (Ref)				1 (Ref)			
	>9.5	16.46 (4.85–55.82)	<0.001	4.81 (0.77–29.85)	0.092	21.76 (10.28–46.09)	<0.001	2.97 (0.85–10.43)	0.089
Creatinine, μmol/L	≤100	1 (Ref)				1 (Ref)			
	>100	1.90 (0.90–3.99)	0.092	0.93 (0.24–3.54)	0.914	6.11 (3.14–11.87)	<0.001	1.34 (0.34–5.24)	0.678
PT, s	≤18	1 (Ref)				1 (Ref)			
	>18	10.59 (1.84–60.81)	0.008	1.16 (0.10–13.29)	0.908	17.00 (5.77–50.06)	<0.001	5.58 (0.84–37.06)	0.075
APTT, s	≤40	1 (Ref)				1 (Ref)			
	>40	7.04 (1.35–36.82)	0.021	6.33 (0.47–85.51)	0.165	6.44 (2.02–20.50)	0.002	0.21 (0.02–2.01)	0.174
D-dimer, mg/L	≤0.6	1 (Ref)				1 (Ref)			
	>0.6	5.50 (2.14–14.12)	<0.001	2.78 (0.86–9.02)	0.089	12.87 (4.99–33.17)	<0.001	3.93 (1.27–12.19)	0.018

*OR, odds ratio; 95%CI, 95% confidence intervals. *Multivariable models were adjusted by age, history of hypertension, coronary artery disease, chronic kidney disease. hs-CRP, high-sensitive C-reactive protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBil, total bilirubin; hs-cTnI, high-sensitive cardiac troponin I; BNP, brain natriuretic peptide; PT, prothrombin time; APTT, activated partial thromboplastin time.*

To an extent, the above findings partly accounted for the potential pathophysiological mechanism of myocardial injury caused by COVID-19, such as direct viral invasion and possibly ischemia-reperfusion injury of the myocardium. Nevertheless, a much larger body of literature suggests that the high degree of systemic inflammation and microvascular thrombosis mediated by the cytokine release syndrome in hospitalized COVID-19 patients may be more principal in the development of myocardial injury (Akhmerov and Marbán, 2020; Colling and Kanthi, 2020). In SARS-CoV-2 infection, the abnormal release of proinflammatory factors could cause endothelial cell apoptosis, resulting in immunopathogenic damage to the cardiovascular system (Teuwen et al., 2020). These factors may shift the balance of coagulation toward a procoagulant and prothrombotic state (Corrales-Medina et al., 2013). Consistently, our study demonstrated that both the male and female patients with myocardial injury presented abnormal inflammation and coagulation stress, as suggested by the higher levels of hs-CRP and D-dimer, and developed elevated leukocyte counts and neutrophil percentages. We also observed that the

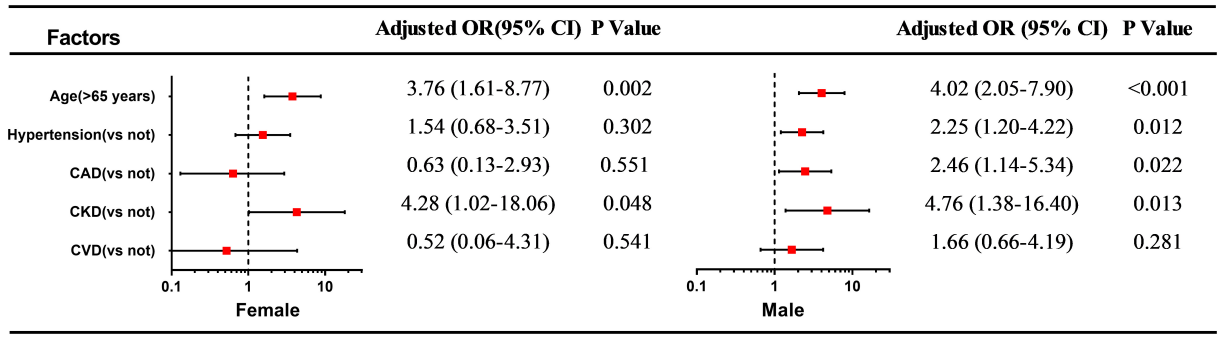

FIGURE 2 | Forest plot of multivariate logistic regression analysis of age and comorbidities associated with myocardial injury in female and male COVID-19 inpatients. OR: odds ratio, 95%CI: 95% confidence intervals.

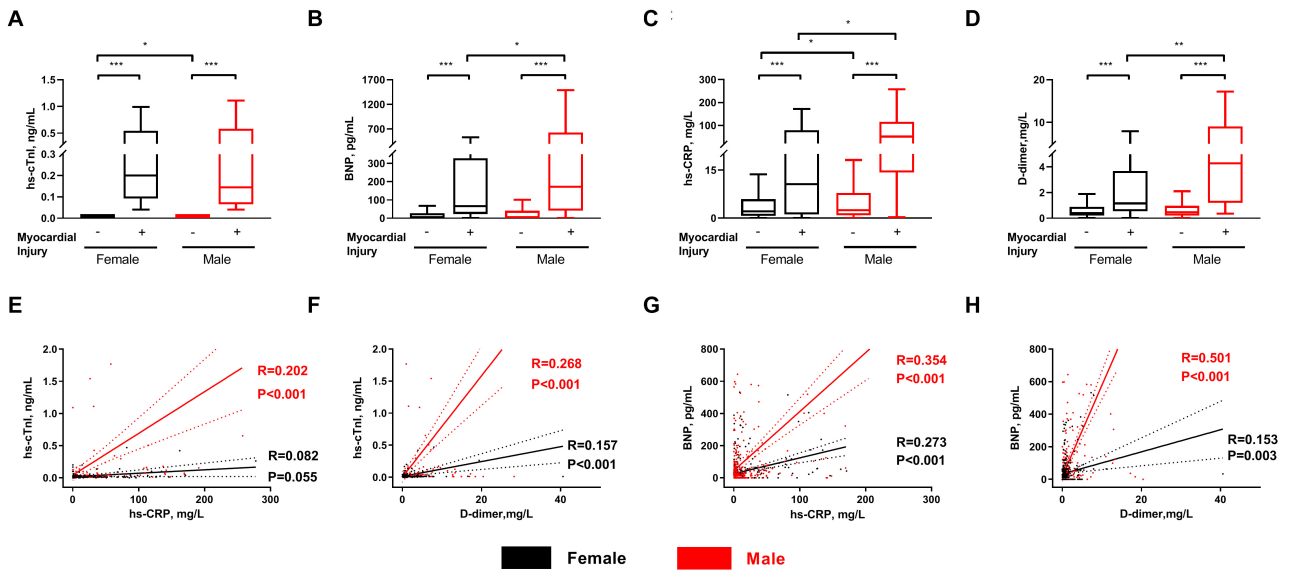

FIGURE 3 | Comparison and correlation of the laboratory testings in COVID-19 inpatients with and without myocardial injury. Comparison of the level of **(A)** hs-cTnI, **(B)** BNP, **(C)** hs-CRP, and **(D)** D-dimer between females and males with and without myocardial injury; Correlation between hs-CRP with hs-TnI **(E)**, D-dimer with hs-TnI **(F)**, hs-CRP with BNP **(G)**, and D-dimer with BNP **(H)** in females and males. Hs-cTnI: high-sensitive cardiac troponin I; BNP: brain natriuretic peptide, hs-CRP: high-sensitive C-reactive protein. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

hemoglobin level was decreased in those with myocardial injury. Taking into account the oxygen-carrying capacity of hemoglobin and cardiac oxygen metabolism imbalance, the latter may be of particular significance in the development of myocardial injury and early prediction of disease prognosis.

Furthermore, our findings add value to those of previous studies by demonstrating that the levels of hs-CRP and D-dimer in men with myocardial injury were almost five and threefold higher than those in the women, respectively. The sexual dimorphism in the hyperinflammatory state may be mediated by different innate and adaptive immune responses based on sex chromosomes (Klein and Flanagan, 2016; Schurz et al., 2019). A large number of immune-related genes located in the X chromosome confer upon women a stronger degree of immune recognition and a higher elimination rate of pathogenic agents (Schurz et al., 2019). As observed in a study that enrolled 331 COVID-19 patients, critically ill female patients have significantly higher levels of SARS-CoV-2 IgG antibodies than their male counterparts (Zeng et al., 2020).

Sex Differences in the Association Between hs-CRP, D-Dimer, and Cardiac Biomarkers

Interestingly, our study supports the notion of the presence of an independent risk relationship of the inflammatory response and coagulation disorder with myocardial injury and cardiac dysfunction in male rather than female patients. It indicated that the men experienced more severe COVID-19 infection, and were more susceptible to inflammation and coagulation stress. Angiotensin-converting enzyme 2 (ACE2) mediates the entry of the virus into host cells by binding with the virus spike

protein. However, this process results in the downregulation of ACE2 as well as uncontrolled renin-angiotensin-aldosterone system activation and further myocardial adverse outcomes (Nishiga et al., 2020). Of note, the ACE2 gene, located on the X chromosome, might experience differences in methylation with sex-chromosome activation (Ambrosino et al., 2020), which probably increased the possibility of sex-oriented susceptibility of myocardial injury.

Meanwhile, women have a higher level of estrogen, which enhances the level of ACE2 activity and expression in a concentration-dependent manner (Rice et al., 2004), upregulates the expression of angiotensin-(1–7) and prompts vasodilation, NO release and reduced smooth muscle cell proliferation (Ji et al., 2008). Estrogen as well exhibits a protective effect against the vascular endothelial injury caused by inflammation (Chakrabarti et al., 2014). Under oxidative stress, estrogen reduces the rate of reactive oxygen species generation by specific posttranslational modifications in the mitochondrial enzymes, inducing a lower rate of myocardial injury in women (Lagranha et al., 2010). Accordingly, we hypothesized that the inflammation reaction and coagulation state vary according to sex and female-specific protective mechanisms, probably mediating sex differences in the incidence of myocardial injury and resulting in sex differences in the incidence of adverse outcomes in COVID-19 patients.

Limitations

Our study has some limitations. Firstly, data on virus antibodies and proinflammatory cytokines (e.g., interleukin [IL]-1, IL-6, IL-8 and tumor necrosis factor-α) were not available, which would provide a proper insight into the pathophysiological stage of the myocardial injury from viral infection to the immune reaction. Moreover, the widespread application of echocardiography was limited due to the rapid progress of the emergency in Wuhan and the consideration of biosafety protection measures for hospital staff. Echocardiographic data were available only in partial subjects and were not analyzed in our retrospective research. Next, this study had a single-center design; our findings require validation in further rigorous prospective studies. Fourthly, our study was retrospective in nature and could only speculate the biological relationship between sex differences and myocardial injury on the basis of our evidence and that of previous studies.

CONCLUSION

Our results suggest that the incidence of myocardial injury in COVID-19 patients is sex-dependent, predominantly in association with a higher degree of inflammation and coagulation disorder in men. These findings may provide a reasonable explanation for the observed sex differences in the adverse outcomes in COVID-19 patients and a theoretical basis for sex-based clinical trials and management.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Ethics Committee, Huoshenshan Hospital (No. HSSLL023). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

RC, CL, and JY contributed to the study design and drafting of the manuscript. LH revised the final manuscript. HT, XD, LZ, and PL contributed to the data collection. RC, CL, JY, YY, YS, RZC, XG, JK, FY, and CH analyzed the data. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.632123/full#supplementary-material

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Predictive Value of the Age, Creatinine, and Ejection Fraction (ACEF) Score in Patients With Acute Fulminant Myocarditis

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Objective: Patients with acute fulminant myocarditis often have more adverse cardiovascular events and higher mortality. The purpose of this study was to evaluate the usefulness of age, creatinine, and left ventricular ejection fraction (ACEF score), in determining the risk that acute fulminant myocarditis will lead to serious cardiovascular events, death, and cardiac dysfunction.

Methods: We retrospectively reviewed the demographics, laboratory tests, medications, echocardiographic examinations, in-hospital clinical outcomes, major adverse cardiovascular events (MACE), and survival rate at 1 year in the medical records of 220 consecutive subjects suffering from acute fulminant myocarditis from January 2013 to June 2019.

Results: Two hundred twenty patients were divided into a survivor group and a non-survivor group. This study found that patients in the non-survivor group were older, had higher heart rates, and had more serious injuries to multiple organ functions. A high ACEF score at admission was independently associated with an unfavorable prognosis, and it was a predictor of in-hospital mortality. The current analysis extends the predictive performance of the ACEF scores at 30 days by evaluating echocardiographic data as applied to survivors of fulminant myocarditis and cumulative rates of MACE at 1 year. The results indicated that patients with high ACEF scores had poor recovery of cardiac function, and higher rates of MACE, all-cause death, and heart failure at 1 year than the low-ACEF group.

Conclusion: The ACEF score was identified as an effective predictor of poor in-hospital outcomes, worse cardiac recovery after 30 days, and higher rates of MACE, all-cause death, and heart failure at 1 year in patients who had acute fulminant myocarditis. These data suggest that its predictive accuracy means the ACEF score could be used to assess the prognosis of patients with acute fulminant myocarditis.

Keywords: age, creatinine, left ventricular ejection fraction, risk prediction, fulminant myocarditis

INTRODUCTION

Acute myocarditis is an autoimmune inflammation of the myocardium to the possible sources with the expression of various clinical manifestations, myocardial damage, hemodynamic disorders, severe arrhythmias, and unfavorable prognosis (McCarthy et al., 2000; Eckart et al., 2004; Gupta et al., 2008; Sharma et al., 2019). Despite the considerably high risk of heart attack, life-threatening arrhythmias and shock, patients with acute fulminant myocarditis might recover and survive longer if they live through the acute phase and if their cardiac function recovers within 1 month (McCarthy et al., 2000; Ammirati et al., 2017; Sharma et al., 2019). Thus, early recognition and risk stratification would lower the in-hospital mortality in such patients if impressive advances in medical therapeutic measurements and aggressive mechanical circulatory support were used earlier (Diddle et al., 2015; Li et al., 2019).

A number of risk factors have been associated with in-hospital mortality and longer-term outcomes in patients who suffer from acute fulminant myocarditis, especially renal dysfunction and impaired cardiac function (Yang et al., 2012; Xu et al., 2018). However, until now, there have been few simple and effective tools to evaluate the in-hospital and 30 day prognosis and long-term survival in patients after acute fulminant myocarditis. The age, creatinine, and left ventricular ejection fraction (ACEF) score was originally developed to predict 1 year mortality in patients who survived for >30 days after acute myocardial infarction (Lee et al., 2015) and to assess mortality risk in elective cardiac operations (Ranucci et al., 2009). Its use has subsequently been extended to other clinical conditions, including acute coronary syndrome, infective endocarditis, and transcatheter aortic valve implantation (Di Serafino et al., 2014; Arai et al., 2015; Stähli et al., 2018; Wei et al., 2019). However, the prognostic value of the ACEF score in patients with acute fulminant myocarditis has not been evaluated. In line with this notion, this study aimed to determine whether the ACEF score is associated with mortality and to investigate the prognostic value of the ACEF score for patients with fulminant myocarditis. The results might help clinical physicians in clinical assessment and decision-making.

MATERIALS AND METHODS

Study Population

This was a retrospective, single-center observational study of 225 patients diagnosed with fulminant myocarditis who were admitted to a cardiac intensive care unit between January 2013 and June 2019. The procedures of the study conformed to the Helsinki Declaration with regard to ethical principles, and use of the participants' data was in accordance with the ethical standards of the institutional committees. All authors confirmed that each patient's information was identified by an alias. The data were collected and divided into survivor and non-survivor groups. The patients standard transthoracic echocardiography at admission.

Data Collection

Each patient's clinical characteristics, clinical manifestations, laboratory examinations, echocardiographic data, and ACEF score were collected and analyzed. The clinical characteristics included gender, age, prior hypertension, prior diabetes mellitus, alcohol use, and smoking. The clinical manifestations referred to heart rate, mean arterial blood pressure, respiratory symptoms, alimentary symptoms, fever, chest tightness or dyspnea, chest pain, and neurological symptoms. Laboratory biomarkers, including white blood cell count (WBC counts, reference value 3.5–9.5 × 10E12/L), hemoglobin (reference value 115–160 g/L), MB isoenzyme of creatine kinase (CK-MB, reference value 0–24 U/L), total bilirubin (normal range 3.4–17.1 μmol/L), and serum creatinine (Scr, normal range 0.7–1.5 mg/dL), were measured at admission. Cardiac structure and function were evaluated based on echocardiographic changes in left atrium dimensions (LAd), left ventricular end systolic dimensions (LVESd), left ventricular end diastolic dimensions (LVEDd), left ventricular ejection fraction (LVEF), pericardial effusion, weakening motion of the ventricular wall, and valve regurgitation. These echocardiographic data were measured with M-mode and two-dimensional Doppler echocardiography. The ACEF score was calculated according to the following formula: ACEF = age/LVEF+1 (if creatinine was >2.0 mg/dL) (Ranucci et al., 2009).

The variables related to incidence of death in subjects were analyzed using multivariate logistic regression to identify independent predictors. All enrolled patients were then divided into two groups according to their ACEF score at admission: a low ACEF score group (ACEF score ≤ 1.43) and a high ACEF score group (ACEF score > 1.43). The clinical characteristics, laboratory examinations, and echocardiography at admission were examined according to different levels of ACEF scores. In addition, therapeutic treatments and strategies, as well as in-hospital complications [shock, New York Association (NYHA class), multiple organ failure, and death] between the group with low ACEF scores and the group with high ACEF scores were analyzed. The therapeutic treatments and strategies included intravenous injection of medication (vitamin C, immunoglobulin, methylprednisolone, diuretics, dopamine, norepinephrine, inotropic agents), oral administration of medication (renin-angiotensin system inhibitors, beta-receptor blockers, aldosterone antagonists), and other medical assistance such as temporary pacemaker, ventilator support, intra-aortic balloon pump (IABP), continuous renal replacement therapy (CRRT), and extracorporeal membrane oxygenation (ECMO). In addition, for survivors after 1 month, the echocardiographic data between the low-ACEF group and the high-ACEF group were compared. Patients with fulminant myocarditis were followed up for 1 year. Major adverse cardiovascular events (MACE) were defined as the composite of all-cause death, heart failure, and readmission. The 1 year all-cause death and the data of clinical follow-up were obtained by reviewing medical records and through telephone interviews with patients on.

Statistical Analysis

Statistical analysis was performed using the SPSS software package (version 19.0, SPSS, United States). Continuous variables were expressed as mean ± standard deviation when normally distributed, and they were compared using the independent-sample *t*-test or Mann Whitney *U*-test. Otherwise, comparison

was made using the Wilcoxon test and shown as median (quartile range). Categorical variables were presented as numbers (percentages), and they were compared with Pearson's chi-square test or Fisher's exact test. Multivariate logistic regression was performed to determine independent predictors of in-hospital death in the subjects. The accuracy of the ACEF score in predicting mortality was assessed using receiver operating characteristic (ROC) curve analysis. Through ROC curve analysis, the optimum cut-off ACEF value was determined as the point of the highest Youden index (sensitivity + specificity − 1). Patients were categorized into two groups according to the statistical ACEF score: the low-ACEF group and the high-ACEF group. The 1 year rates of cumulative MACE events were evaluated by the Kaplan-Meier method, and the difference between groups was assessed by log-rank test in patients with acute fulminant myocarditis. A $p < 0.05$ (two-sided) was defined as statistically significant.

RESULTS

Patients' Clinical Characteristics, Performance, Laboratory Findings, Echocardiographic Examination, and ACEF Scores

The 225 patients with fulminant myocarditis were enrolled, and 5 patients were excluded because of incomplete data. Among the remaining 220 patients, 24 (10.91%) died in hospital and were classified as a non-survivor group. The other 196 patients were classified into a survivor group. The baseline characteristics, clinical manifestations, laboratory data, echocardiographic measurements, and ACEF scores at admission are presented in **Table 1**. Differences between the two groups in gender, proportion of prior medical histories, mean arterial blood pressure, frequency of clinical presentation, and hemoglobin level did not reach statistical significance. With respect to echocardiographic data (LAd, LVEDd, pericardial effusion, weakening motion of the ventricular wall, and valve regurgitation), patients who suffered acute fulminant myocarditis in the non-survivor group had no significant difference when compared with patients in the survivor group (**Table 1**).

The patients with fulminant myocarditis in the non-survivor group were older [52.63 ± 18.08 vs. 35.00 (24.25∼49.75)], and they had higher heart rates (115.58 ± 28.90 vs. 80.67 ± 23.76 bpm) than the survivors who complicated acute fulminant myocarditis. Patients who did not survive after fulminant myocarditis had higher WBC counts [13.77 ± 8.82 vs. 8.61 (6.10∼11.89) × 10E12/L), CK-MB [95.37 ± 66.45 vs. 25.61 (9.21∼63.84) U/L], total bilirubin [25.26 ± 20.46 vs. 12.70 (9.20∼17.80) μmol/L), and serum creatinine [1.66 (0.95∼1.91) vs. 0.83 (0.65∼1.05) mg/dL] at admission compared to survivors. In addition, patients with acute fulminant myocarditis who did not survive had a significantly higher mean LVESd [40.29 ± 6.81 vs. 36.00 (32.00∼40.00) mm], and a dramatically lower LVEF [0.34 ± 0.08 vs. 0.51 (0.40∼0.61)] in comparison with the patients who survived. Importantly, ACEF scores were higher in patients in the non-survivor group than in the survivor group (**Table 1**).

TABLE 1 | Comparison of the clinical features and the ACEF score in patients with acute fulminant myocarditis.

Variables	Survivor (*n* = 196)	Non-survivor (*n* = 24)	*P*-value
Clinical characteristics			
Gender (male) [*n* (%)]	123 (62.76%)	15 (62.5%)	0.981
Age (years)	35.00 (24.25∼49.75)	52.63 ± 18.08*	0.001
Prior hypertension [*n* (%)]	32 (16.33%)	6 (25.00%)	0.267
Prior diabetes mellitus [*n* (%)]	13 (6.63%)	2 (8.33%)	0.671
Alcohol [*n* (%)]	22 (11.22%)	3 (12.50)	0.741
Smoking [*n* (%)]	44 (22.45%)	4 (16.67%)	0.517
Heart rate (bpm)	80.67 ± 23.76	115.58 ± 28.90*	0.000
Mean arterial blood pressure (mmHg)	80.48 ± 13.33	79.28 ± 23.70	0.809
Clinical manifestation			
Respiratory symptom [*n* (%)]	63 (32.14%)	11 (32.14%)	0.180
Alimentary symptom [*n* (%)]	47 (23.98%)	9 (37.5%)	0.151
Fever *n* [*n* (%)]	111 (56.63%)	17 (70.83%)	0.183
Chest tightness or dyspnea [*n* (%)]	137 (69.90%)	20 (83.33%)	0.169
Chest pain [*n* (%)]	54 (27.55%)	5 (20.83%)	0.483
Neurological symptom (syncope) [*n* (%)]	36 (18.37%)	7 (29.17%)	0.161
Laboratory examination			
White blood cell counts (×10 E12/L)	8.61 (6.10∼11.89)	13.77 ± 8.82*	0.041
Hemoglobin (g/L)	131.51 ± 20.65	137.17 ± 27.47	0.348
CK-MB (U/L)	25.61 (9.21∼63.84)	95.37 ± 66.45*	0.000
Total bilirubin (μmol/L)	12.70 (9.20∼17.80)	25.26 ± 20.46*	0.028
Serum creatinine (mg/dL)	0.83 (0.65∼1.05)	1.66 (0.95∼1.91)*	0.000
Echocardiographic parameters			
LAd (mm)	35.85 ± 6.00	37.86 ± 7.59	0.148
LVESd (mm)	36.00 (32.00∼40.00)	40.29 ± 6.81*	0.002
LVEDd (mm)	49.0 (46.00∼53.00)	49.36 ± 5.31	0.961
LVEF	0.51 (0.40∼0.61)	0.34 ± 0.08*	0.000
Pericardial effusion [*n* (%)]	66 (33.67%)	11 (45.83%)	0.238
Weakening motion of the ventricular wall [*n* (%)]	108 (55.10%)	17 (70.83%)	0.142
Valve regurgitation [*n* (%)]	72 (36.73%)	14 (58.33%)	0.105
ACEF score	0.74 (0.49∼1.15)	2.14 ± 0.94*	0.000

*Values are given as mean ± standard deviation, median and interquartile range or number and percentages. *P < 0.05 (survivor group vs. non-survivor group).*
CK-MB, MB isoenzyme of creatine kinase; LAd, left atrium diameter; LVESd, left ventricular end-systolic diameter; LVEDd, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; ACEF score, the age, creatinine, and ejection fraction score.

Clinical Outcomes and Predictors of In-Hospital Death

Six risk factors (heart rate, WBC count, CK-MB, total bilirubin, LVESd, and ACEF) were ranked for predicting in-hospital death. Multivariate logistic regression demonstrated that the

ACEF score [odds ratio (OR): 4.499; 95% confidence interval (CI): (0.960–1.061); $p < 0.000$] was confirmed to be a strong independent predictor of in-hospital death in patients with acute fulminant myocarditis in contrast to other risk factors (**Table 2**). The ACEF score displayed good prognostic information for in-hospital mortality based on ROC curve analysis, and the area of ROC was 0.871 (**Figure 1**).

Evaluation of Clinical Characteristics, Laboratory Tests, Echocardiographic Findings on Admission, In-Hospital Medical Treatments, and Clinical Complications

A recent study reported on the relationship between ACEF scores and all-cause mortality in patients with acute coronary syndrome (Stähli et al., 2018). Based on ROC curve analysis, it was determined that an ACEF score of 1.43 was the optimum cut-off value, since it had the highest Youden index. Therefore, the patients were reclassified into two groups according to their ACEF scores. A low ACEF score ($\leq$1.43, $n = 170$) indicated a low risk of death, and a high ACEF score ($>$1.43, $n = 50$) indicated a high risk of death.

TABLE 2 | The predictors of in-hospital mortality in patients with acute fulminant myocarditis by multivariate logistic regression analysis.

Variables	Odds ratio (95% CI)	*P*-value
Heart rate (bpm)	1.028 (0.997–1.060)	0.081
White blood cell counts (×10 E12/L)	1.019 (0.930–1.118)	0.685
Primary CK-MB (U/L)[a]	1.006 (0.998–1.015)	0.159
Primary total bilirubin	1.009 (0.960–1.061)	0.718
Left ventricular end-systolic dimension (mm)	0.982 (0.892–1.080)	0.704
ACEF score[b]	4.499 (0.960–1.061)	0.000*

*$P < 0.05$.
[a]*CK-MB, MB isoenzyme of creatine kinase.*
[b]*ACEF score, the age, creatinine, and ejection fraction score.*

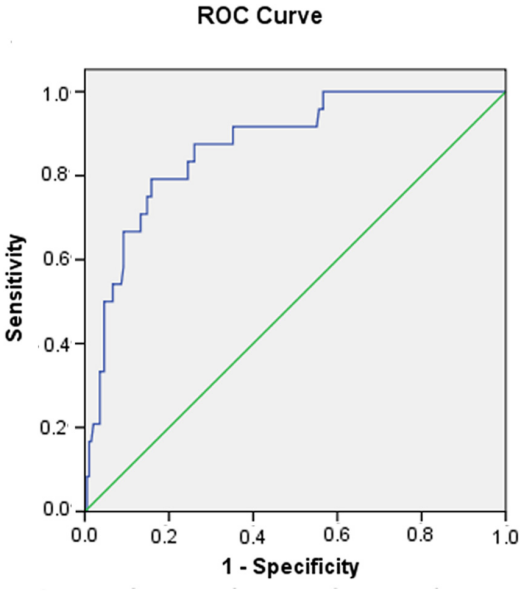

FIGURE 1 | Receiver operating characteristic (ROC) curve of the ACEF score in predicting in-hospital death in patients with acute fulminant myocarditis.

Gender, frequency of alcohol use, and frequency of smoking had no significant difference between the low-ACEF group and the high-ACEF group. The patients in the high-ACEF group were older, and more of them had a history of hypertension and diabetes. This indicated that older patients or patients with more clinical diseases might have a higher risk of death.

The differences between the low and high ACEF groups in echocardiographic measurements on admission were analyzed. There was no statistically significant difference between the two groups with regard to pericardial effusion, weakening ventricular wall motion, and valve regurgitation. By contrast, patients with fulminant myocarditis in the high-ACEF group had higher LAd (39.51 ± 6.65 vs. 35.05 ± 5.68 mm, $p < 0.05$), LVESd [41.40 ± 6.22 vs. 34.00 (31.00∼39.00) mm, $p < 0.05$), and LVEDd [50.94 ± 5.30 vs. 48.00 (46.00∼51.00) mm, $p < 0.05$], but a notable decrease in LVEF [0.37 ± 0.09 vs. 0.55 (0.42∼0.62), $p < 0.05$) than the low-ACEF group (**Table 3**). These results demonstrated that patients with high ACEF scores had more serious cardiac dysfunction than the patients with low ACEF scores (**Table 3**).

Next, we evaluated the treatments and clinical complications in both groups. Patients in the high-ACEF group had higher rates of prescriptions for diuretics, dopamine, and norepinephrine. They also had a greater need for inotropic agents, ventilator supports, IABP, CRRT, and ECMO than those in the low-ACEF group. This implied that the patients in the high-ACEF group had more serious conditions. By contrast, no significant differences were observed between the two groups with respect to treatment with renin-angiotensin system inhibitors, beta-receptor blockers, aldosterone antagonists, vitamin C, immunoglobulins, methylprednisolone, and temporary use of pacemakers. These results demonstrated that the patients in the high-ACEF group needed more medical support and were in worse condition than the patients in the low-ACEF group (**Table 3**).

The patients with fulminant myocarditis in the high-ACEF group were more likely to develop clinical complications [shock, NYHA III-IV, ventricular tachycardia/ventricular fibrillation (VT/VF), multiple organ failure, and death] than the patients in the low-ACEF group. This indicated that patients in the high-ACEF group were at greater risk of serious adverse cardiac events. Importantly, the mortality rate of patients with acute fulminant myocarditis was 38.0% in the high-ACEF group and 2.94% in the low-ACEF group (**Table 3**).

Evaluation of Electrocardiographic Data at 30 Days and the Cumulative Rates of MACE at 1 Year in Patients With Acute Fulminant Myocarditis

The echocardiographic measurements 1 month after discharge in survivors were compared according to their ACEF scores.

TABLE 3 | Summary of the clinical features according to ACEF score in patients with acute fulminant myocarditis.

	ACEF score ≤ 1.43 (*n* = 170)	ACEF score > 1.43 (*n* = 50)	*P*-value
Clinical characteristics			
Gender (male) [*n* (%)]	107 (62.94%)	31 (62.00%)	0.904
Age (years)	34.64±14.34	59.34±15.64*	0.000
Prior hypertension [*n* (%)]	16 (9.41%)	22 (44.00%)*	0.000
Prior diabetes mellitus [*n* (%)]	7 (4.12%)	8 (16.00%)*	0.009
Alcohol [*n* (%)]	18 (10.59%)	7 (14.00%)	0.473
Smoking [*n* (%)]	35 (20.59%)	13 (26%)	0.415
Laboratory examination			
White blood cell counts (×10 E12/L)	8.12 (5.97∼11.16)	13.62 ± 8.19*	0.000
Hemoglobin (g/L)	132.08 ± 21.66	132.20 ± 20.99	0.973
CK-MB (U/L)	24.92 (8.69∼56.69)	75.64 ± 59.00*	0.001
Total bilirubin (μmol/L)	13.77 ± 7.27	15.80 (10.70∼28.70)*	0.001
Serum creatinine (mg/dL)	0.82 ± 0.24	1.75 (1.29∼2.30)*	0.000
Echocardiographic data on admission	***n* = 170**	***n* = 50**	
LAd (mm)	35.05 ± 5.68	39.51 ± 6.65*	0.000
LVESd (mm)	34.00 (31.00∼39.00)	41.40 ± 6.22*	0.000
LVEDd (mm)	48.00 (46.00∼51.00)	50.94 ± 5.30*	0.005
LVEF	0.55 (0.42∼0.62)	0.37 ± 0.09*	0.000
Pericardial effusion [*n* (%)]	52 (30.59%)	14 (28.00%)	0.726
Weakening motion of ventricular wall [*n* (%)]	76 (44.71%)	22 (44.00%)	0.930
Valve regurgitation [*n* (%)]	50 (29.41%)	22 (44.00%)	0.053
Medical treatments	**(*n* = 170)**	**(*n* = 50)**	
Renin-angiotensin system inhibitor [*n* (%)]	81 (47.65%)	22 (44.00%)	0.650
Beta receptor blocker [*n* (%)]	89 (52.35%)	19 (38.00%)	0.074
Aldosterone antagonist [*n* (%)]	59 (34.71%)	18 (36.00%)	0.866
Vitamin C [*n* (%)]	153 (90.00%)	44 (88.00%)	0.000
Immunoglobulin [*n* (%)]	101 (59.41%)	34 (68.00%)	0.273
Methylprednisolone [*n* (%)]	123 (72.35%)	38 (76.00%)	0.609
Diuretics [*n* (%)]	60 (35.29%)	38 (76.00%)*	0.000
Dopamine [*n* (%)]	30 (17.65%)	25 (50.00%)*	0.000
Norepinephrine [*n* (%)]	19 (11.18%)	23 (46.00%)*	0.000
Inotropic agent [*n* (%)]	8 (4.71%)	21 (42.00%)*	0.000
Temporary pacemaker [*n* (%)]	32 (18.82%)	8 (16.00%)	0.649
Ventilator support [*n* (%)]	19 (11.18%)	26 (52.00%)*	0.000
Intra-aortic balloon pump [*n* (%)]	14 (8.24%)	20 (40.00%)*	0.000
CRRT [*n* (%)]	2 (1.18%)	17 (34.00%)*	0.000
ECMO [*n* (%)]	1 (1.43%)	4 (8.00%)*	0.002
Clinical complication			
Shock [*n* (%)]	35 (20.59%)	34 (68.00%)*	0.000
NYHA			
Grade I-II [*n* (%)]	112 (65.88%)	17 (34.00%)*	0.000
Grade III-IV [*n* (%)]	58 (34.12%)	33 (66.00%)*	0.000
VT/VF [*n* (%)]	14 (8.24%)	19 (38.00%)*	0.000
Multiple organ failure [*n* (%)]	47 (27.65%)	38 (76.00%)*	0.000
Death [*n* (%)]	5 (2.94%)	19 (38.00%)*	0.000

*Values are presented as mean ± standard deviation, median and interquartile range or number and percentages. *P < 0.05 (ACEF score > 1.43 vs. ACEF score ≤ 1.43). ACEF, the age, creatinine, and left ventricular ejection fraction; CK-MB, MB isoenzyme of creatine kinase; Lad, left atrium diameter; LVESd, left ventricular end-systolic diameter; LVEDd, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; CRRT, continuous renal replacement therapy; ECMO, extracorporeal membrane oxygenation; VT/VF, ventricular tachycardia/ventricular fibrillation.*

Patients in the high-ACEF group had markedly higher LAd [39.70 ± 6.34 vs. 35.24 ± 5.10 mm, $p < 0.05$], LVESd [36.30 ± 6.45 vs. 32.00 (30.00∼35.00) mm, $p < 0.05$)], and LVEDd [51.53 ± 5.24 vs. 49.00 (45.00∼52.00) mm, $p < 0.05$)], but remarkably lower LVEF [0.55 ± 0.98 vs. 0.62 (0.58∼0.68), $p < 0.05$]. These data also indicated greater prevalence of weakening motion of the ventricular wall and valve regurgitation in the high-ACEF group. These results showed that high ACEF

TABLE 4 | Echocardiographic data at 30 day in patients with acute fulminant myocarditis.

Echocardiographic data	ACEF score ≤ 1.43 (*n* = 165)	ACEF score > 1.43 (*n* = 31)	*P*-value
LAd (mm)	35.24 ± 5.10	39.70 ± 6.34	0.088
LVESd (mm)	32.00 (30.00∼35.00)	36.30 ± 6.45*	0.001
LVEDd (mm)	49.00 (45.00∼52.00)	51.53 ± 5.24*	0.01
LVEF	0.62 (0.58∼0.68)	0.55 ± 0.98*	0.001
Pericardial effusion [*n* (%)]	33 (20.00%)	6 (19.35%)	0.934
Weakening motion of the ventricular wall [*n* (%)]	44 (26.67%)	22 (70.97%)*	0.000
Valve regurgitation [*n* (%)]	42 (25.45%)	19 (61.29%)*	0.000

*Values are presented as mean ± standard deviation, median and interquartile range or number and percentages. *P < 0.05 (ACEF score > 1.43 vs. ACEF score ≤ 1.43).*
ACEF, the age, creatinine, and left ventricular ejection fraction; LAd, left atrium diameter; LVESd, left ventricular end-systolic diameter; LVEDd, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction.

scores were closely correlated with myocardial recovery at 1 month in patients with acute fulminant myocarditis (**Table 4**).

Those patients were followed up for 1 year. Among them, 160 patients in the low-ACEF group (ACEF ≤ 1.43) and 49 patients in the high-ACEF group (ACEF > 1.43) were included while 11 patients were lost in the follow-up period. The rates of MACE, all-cause death, and cardiac failure attack at 1 year were remarkably higher in the high-ACEF group compared to those patients with low ACEF scores (**Figure 2**). These data clearly demonstrated the value of the ACEF score for predicting 1 month and 1 year outcomes in patients with acute fulminant myocarditis.

DISCUSSION

This study successfully analyzed the differences in clinical presentation of patients with acute fulminant myocarditis, and it established one simple and precise ACEF score assessment tool. It found that patients with high ACEF scores had more severe disease conditions, required more medical treatments, and possibly had higher clinical complications and mortality rates than the patients with low ACEF scores. In addition, ACEF scores demonstrated a strong ability to predict recovery of cardiac function in 30 day survivors and the risk of MACE, all-cause death and cardiac failure attack in patients with acute fulminant myocarditis. Thus, the ACEF score was shown to be a valuable predictor for patients undergoing acute fulminant myocarditis in terms of assessing their risk of in-hospital mortality and long-term prognosis.

A total of 220 patients with acute fulminant myocarditis were included in the present study. The patients with acute fulminant myocarditis in the non-survivor group presented with a broad spectrum of symptoms and severe cardiac dysfunction, and they needed more medical treatments and circulatory support or heart transplantation. Our critical findings were in accordance with previous results (Ammirati et al., 2018; Veronese et al., 2018). Early risk stratification contributed to patients with acute fulminant myocarditis due to high short-term and long-term mortality. In previous studies, many risk factors were found to be related to poor prognosis for developing fulminant myocarditis in patients, especially echocardiographic data and kidney injury (Yang et al., 2012; Xu et al., 2018), and prolonged PR interval and widened QRS complex (Sun et al., 2017). The echocardiographic features of myocarditis in the non-survivor group were often non-specific, but evaluating heart function with echocardiographic data was helpful in determining prognosis. In the current study, the patients in the non-survivor group were older, had higher serum creatinine, and had lower LVEF than the patients in the survivor group. Thus, the predictive ability of a single factor was proven to be insufficient. Among many parameters (heart rate, WBC count, CK-MB, total bilirubin, LVESd, and ACEF), the ACEF score at admission, by incorporating three easily obtainable variables (age, creatinine, and LVEF), was independently associated with an unfavorable prognosis, and it was a predictor of in-hospital mortality in patients with acute fulminant myocarditis.

Early estimation of prognosis in patients with acute fulminant myocarditis is difficult due to limited clinical studies on long-term outcomes (Sharma et al., 2019). This new ACEF score was simpler to establish and more accurate for developing a prognosis for acute fulminant myocarditis. A high ACEF score probably reflected the more serious conditions and worse prognosis of

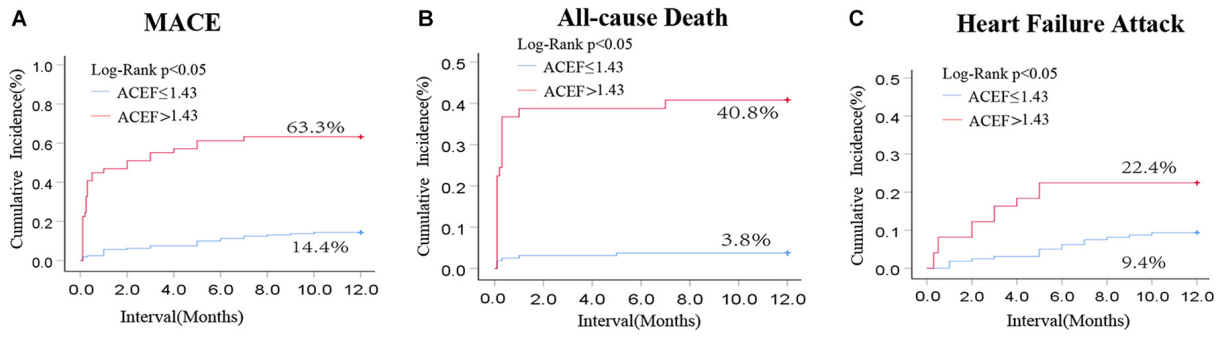

FIGURE 2 | Kaplan-Meier curve for cumulative rates of MACE **(A)**, all-cause rate **(B)**, and heart failure attack **(C)** according to different levels of the ACEF score in patients with cute fulminant myocarditis.

patients with acute fulminant myocarditis. Thus, patients with high ACEF scores may benefit from early invasive management and more aggressive use of hemodynamic support devices. The ACEF score previously was recommended for evaluating mortality risk in cardiac surgery, and it was considered to be an independent predictor for in-hospital and long-term mortality in patients with infective endocarditis (Ranucci et al., 2009; Wei et al., 2019). Moreover, the ACEF score had been used to stratify the risk of 1 year clinical outcome and prognostic impact in 30 day survivors of acute myocardial infarction after percutaneous coronary intervention (Lee et al., 2015; Stähli et al., 2018; Gao et al., 2020). The current study was accomplished by evaluating the predictive ability of the ACEF scores. A higher ACEF score markedly indicated worse clinical course in hospital, a poor recovery of cardiac function at 30 days, and higher rates of MACE and death in patients who suffered from acute fulminant myocarditis. Clinical sepsis produced substantial cardiomyocytes injury which was closely correlated to a reduced peak of intracellular Ca^{2+} sequestration, but no changes in resting intra-cellular Ca^{2+} and Ca^{2+}-transient decay. It is possible that fulminant myocarditis leading to low cardiac output syndrome, shock and life-threatening arrhythmia, might be attributed to alterations in Ca^{2+} transient properties and the mechanical properties (Ren et al., 2002). Consistently, this study determined that it was acceptable to use the ACEF score to predict short-term and long-term outcomes in patients after acute fulminant myocarditis.

LIMITATIONS

Some limitations inherent to the study design should be acknowledged. First, the number of patients referred for acute fulminant myocarditis was rather small. Second, the proposed ACEF score risk categories must be tested in an external validation cohort. Third, although a comprehensive group of variables was used in the multivariate models, not all risk scores developed for the multivariate models were included.

CONCLUSION

In this study, the ACEF score, which incorporates three objectively measurable risk factors (age, creatinine level, and LVEF), is an extremely simple, practical, easy-to-calculate, and user-friendly tool for determining the prognosis in the acute fulminant myocarditis patient population. Furthermore, in contrast to other risk scores, the ACEF score allows for the identification of risk stratification, adverse events, and prognosis, which may further influence management decisions in acute fulminant myocarditis. These findings strengthened the role of the ACEF score and demonstrated that it had better predictive ability and could independently predict clinical adverse events, in-hospital mortality, cardiac function after 1 month of recovery, and 1 year prognosis in patients presenting with acute fulminant myocarditis. The ACEF score provided a novel and effective indicator to stratify the risk for patients with acute fulminant myocarditis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of First Affiliated Hospital of Soochow University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MX and TJ: designing the study. LL, XY, and YG: data collection and analysis. XY, JX, and MX: statistics. MX, LL, and XY: manuscript preparation and writing. MX: English improvement. TJ: funding support. All authors contributed to the article and approved the submitted version.

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Up-Regulation of Glycogen Synthesis and Degradation Enzyme Level Maintained Myocardial Glycogen in Huddling Brandt's Voles Under Cool Environments

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Small mammals exhibit limited glucose use and glycogen accumulation during hypothermia. Huddling is a highly evolved cooperative behavioral strategy in social mammals, allowing adaptation to environmental cooling. However, it is not clear whether this behavior affects the utilization of glycogen in cold environments. Here, we studied the effects of huddling on myocardial glycogen content in Brandt's voles (*Lasiopodomys brandtii*) under a mild cold environment (15°C). Results showed that (1) Compared to the control (22°C) group (CON), the number of glycogenosomes more than tripled in the cool separated group (CS) in both males and females; whereas the number of glycogenosomes increased in females but was maintained in males in the cool huddling group (CH). (2) Glycogen synthase (GS) activity in the CS group remained unchanged, whereas glycogen phosphorylase (GYPL) activity decreased, which mediated the accumulation of glycogen content of the CS group. (3) Both GS and GYPL activity increased which may contribute to the stability of glycogen content in CH group. (4) The expression levels of glucose transporters GLUT1 and GLUT4 increased in the CS group, accompanied by an increase in glucose metabolism. These results indicate that the reduced glycogen degradation enzyme level and enhanced glucose transport may lead to an increase in myocardial glycogen content of the separated voles under cool environment; while the up-regulation of glycogen synthesis and degradation enzyme level maintained myocardial glycogen content in the huddling vole.

Keywords: huddling, low temperature, heart, glycogenosomes, glycogen synthetase, glycogen phosphorylase

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INTRODUCTION

Low temperature is a stress stimulus for mammals, especially for small mammals as their energy requirements are high due to the large surface area to volume ratio. Moreover, when environmental stressors persist for prolonged periods, small animal tissues and organs are more vulnerable to the impact of external environmental temperature (Gilbert et al., 2010; Wei et al., 2018). Hypothermia can lead to a slowed heart rate, decreased blood flow output, and decreased myocardial contraction and relaxation function (Polderman, 2009; Kelly and Nolan, 2010; Tessier and Storey, 2012;

Chavez et al., 2017). As above, the cardiac muscle of small mammals is more susceptible to low external temperatures. Our previous study showed that, in comparison to warm environmental conditions, Brandt's voles (*Lasiopodomys brandtii*) under cool (15°C) conditions exhibit myocardial mitochondrial swelling and crista disruption, as well as decreased adenosine triphosphate (ATP) synthase activity (Wang et al., 2020b). Glucose is the energy supply of mitochondria, and thus changes in mitochondrial function may involve changes in glycogen content in tissues (Hall and Mackay, 1933; Tarnopolsky, 2016; Xu et al., 2020). Altered carbohydrate metabolism during hypothermia in mammals is accompanied by abnormalities in glucose metabolism (Baum et al., 1968; Curry and Curry, 1970; Helman et al., 1984). For example, in rats (Popovic, 1960; Fuhrman and Fuhrman, 1963) and rabbits (Bickford and Mottram, 1960), metabolism of both endogenously and exogenously administered glucose is substantially reduced during hypothermia. Furthermore, exposure to only 4 h of cold temperature (15°C) can lead to an increase in myocardial glycogen content in rats (Steffen, 1988), suggesting that the effects of hypothermia on cardiac muscle may involve the balance between glycogen synthesis and degradation.

Glycogen is a branched polymer of glucose and stores energy in times of nutritional sufficiency for utilization in times of need. Glycogen synthase (GS), a key enzyme for synthesis, polymerizes UDP-glucose to form glycogen granules, with phosphorylated GS (P-GS) being its active state (Palm et al., 2013; Zeqiraj and Sicheri, 2015; Wang et al., 2019). Glycogen phosphorylase (GYPL) is a rate-limiting enzyme that breaks down glycogen granules to glucose (Agius, 2010; Mavrokefalos et al., 2015). The direct pathway of glycogen synthesis requires the transport of glucose into cells by one or several glucose transporters (GLUTs) (Thorens and Mueckler, 2010). GLUT1 is widely distributed and provides basal glucose transport; GLUT4 is up-regulated by insulin and is important in insulin-sensitive tissues, such as skeletal muscle and adipose tissue; and GLUT2 is prominent in the liver and β-cells of the pancreas and admits glucose based on a positive glucose gradient between blood and tissue (Roach et al., 2012). Research on hibernating Daurian ground squirrels (*Spermophilus dauricus*) has shown that the increase in glycogen content in skeletal muscle in winter is mainly due to the maintenance of P-GS and decrease in GYPL protein expression (Wang et al., 2019). Thus, studies on the above factors could help reveal the mechanism related to changes in myocardial glycogen content under cool environments.

Huddling is a social thermoregulatory behavior, defined as the active aggregation of nestled animals. It is a cooperative group behavior, permitting individuals involved in social thermoregulation to minimize heat loss and thereby lower energy expenditure, possibly allowing reallocation of saved energy to other functions (Gilbert et al., 2010; Douglas et al., 2017). It is commonly exhibited in small mammals and birds to reduce heat and energy loss under cold environments (Jefimow et al., 2011; Wojciechowski et al., 2011; Sukhchuluun et al., 2018; Zhang et al., 2018). Research has shown that many mammals, such as degu (*Octodon degus*), Damaraland mole-rat (*Cryptomys damarensis*), and Natal mole-rat (*C. hottentotus natalensis*), huddle when the ambient temperature is lower than 15–20°C, with an energy saving of up to 30% (Kotze et al., 2008; Nunez-Villegas et al., 2014). Research on Eastern pygmy possums (*Cercartetus nanus*) has shown that huddling in mild low temperatures (14°C) can reduce energy consumption by up to 50% (Namekata and Geiser, 2009). The benefits of huddling in energy conservation (Scantlebury et al., 2006; Kotze et al., 2008), local environmental heating (Nowack and Geiser, 2016), and survival (Sealander, 1952) have also been studied in several species. Overall, huddling individuals exhibit increased survival, lower food intake, decreased body mass loss, increased growth rate, more constant body temperature, and reduced metabolic rate (Gilbert et al., 2010). To date, previous studies have primarily focused on morphological and physiological changes in animal bodies under various temperatures. However, no studies have

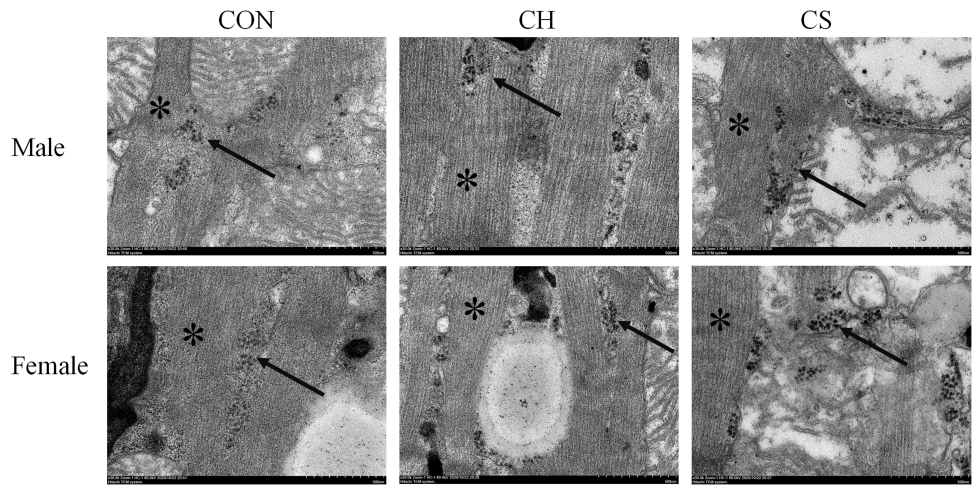

FIGURE 1 | Ultrastructural distribution of myocardial glycogenosomes in Brandt's voles. Arrow indicates glycogenosome. Muscle filaments (see asterisk) was well arranged. Scale bar = 0.5 μm.

reported on changes in myocardial glycogen in mammals under different temperatures.

Brandt's voles are small non-hibernating herbivorous rodents widely distributed among the Inner Mongolian grasslands of Northern China, dry steppe zone of Mongolia, and southeast Baikal region of Russia. They are highly socialized animals that huddle in winter as an adaptation to their harsh habitats (Zhang et al., 2018), which differs substantially from model animals. Research has shown that mild cooling can significantly change the morphology of mitochondria in the cardiac muscle of Brandt's voles (Xu et al., 2019; Wang et al., 2020a). Furthermore, their metabolic rate and thermogenic capacity decrease but activity increases compared with separated individuals under low temperatures, suggesting that huddling is a good strategy for small mammals to cope with cold environments (Sukhchuluun et al., 2018). Glycogen is one of the most important energy supply substances in muscles. However, the role of myocardial glycogen in adaptive huddling has not yet been reported. Therefore, we hypothesized that a cool environment could cause an increase in myocardial glycogen content in Brandt's voles. We also hypothesized that huddling could effectively alleviate this change. To test these hypotheses, we observed the ultrastructure of cardiac muscle in huddling and individual (separated) Brandt's voles under mild temperature differences (normal: 22°C; cool: 15°C) in autumn. We also determined the protein expression levels of glucose transport glycogen synthesis, and glycogen degradation-related signals. We further explored the underlying molecular mechanism related to the effects of a mild cold environment and huddling on changes in myocardial glycogen content.

MATERIALS AND METHODS

Ethics Statement

All procedures followed the Laboratory Animal Guidelines for the Ethical Review of Animal Welfare (GB/T 35892-2018) and

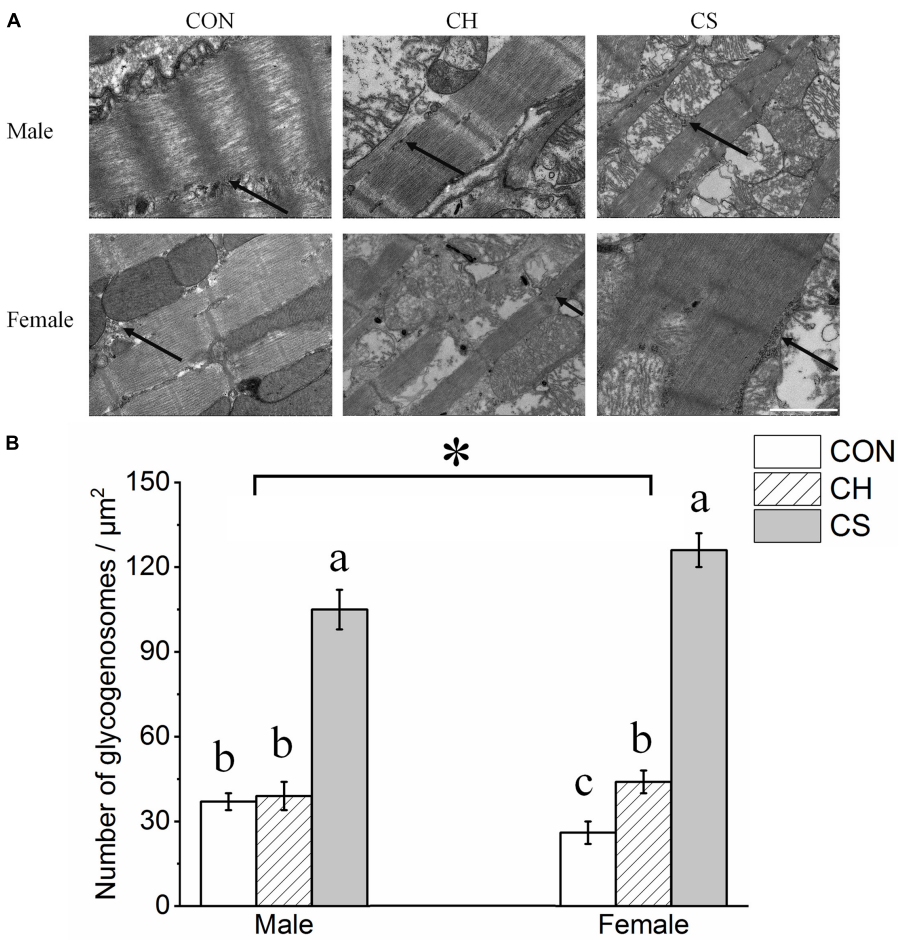

FIGURE 2 | Changes in number of myocardial glycogenosomes in Brandt's voles. **(A)** Myocardial glycogenosomes in three treatment groups. Arrow indicates glycogenosome. Scale bar = 1 μm. **(B)** Bar graph depicting changes in number of glycogenosomes. Values are mean ± SD. Six figures were analyzed in each sample; eight samples were analyzed in each group. CON, control group; CH, cool huddling group; CS, cool separated group. Different letters identify statistically significant differences among temperature treatment groups ($P < 0.05$). *$P < 0.05$ significant differences between males and females.

were approved by the Animal Care and Use Committee of Qufu Normal University (Permit Number: dwsc 2019012).

Animals and Groups

Forty-eight adult voles were captured and housed as described previously (Wang et al., 2020b). The voles were acclimated to laboratory conditions for 2 weeks. They were housed four animals per cage (28 × 18 × 12 cm) at an ambient temperature of 22 ± 2°C, relative humidity of 55 ± 5%, and light/dark regime of 12 h:12 h (light on from 06:00 to 18:00). Food (standard rabbit chow, Pengyue Experimental Animal Breeding Co., Ltd., China) and water was provided *ad libitum* and wood shavings were used as bedding. Based on body weight, a total of 24 males (28–50 g, average 38 g) and 24 female (27–54 g, average 33 g) adult voles were randomly divided into three groups, respectively. Control group (CON): Voles were continuously housed under an ambient temperature of 22 ± 2°C, with four animals in each cage (two males and two females), similar to their normal state in autumn. Cool huddling group (CH): Voles were housed together in a cage (two males and two females) under an ambient temperature of 15°C. The group size (four voles in each cage) ensured most animals remained inactive in a huddle (Sukhchuluun et al., 2018). Cool separated group (CS): Voles were housed individually in cages at an ambient temperature of 15°C. The three treatment groups were maintained under the same relative humidity (55 ± 5%) and light regime (12 h: 12 h light /dark, light on from 06:00 to 18:00). Animal treatment started in late September and lasted 8 weeks (Wang et al., 2020b).

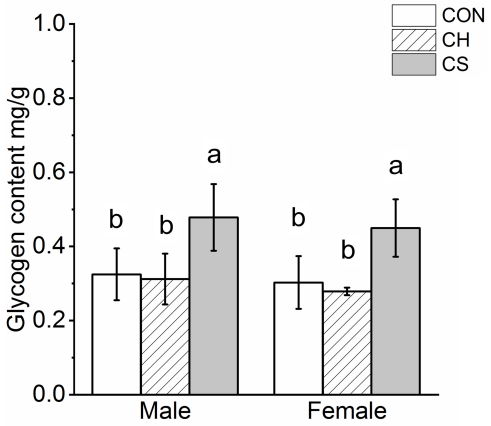

FIGURE 3 | Changes in content of myocardial glycogenosomes in Brandt's voles. Glycogen levels were normalized by cell protein concentration measured using the BCA assay. Different letters identify statistically significant differences among temperature treatment groups ($P < 0.05$). CON, control group; CH, cool huddling group; CS, cool separated group.

Sample Preparation

All animals were sacrificed by CO_2 asphyxiation between 08:00 and 11:00 a.m. on the last day of the experiment (Sukhchuluun et al., 2018; Wang et al., 2020b). After the rapid removal of cardiac muscle, portions of the ventricles were immediately excised and fixed in glutaraldehyde. Specimens were fixed in 1% osmium tetroxide in the same buffer, dehydrated with a graded series of ethanol, and embedded in epoxy resin. The remaining cardiac muscle was frozen in liquid nitrogen and stored at −80°C. All procedures were carried out in accordance with the approved guidelines.

Transmission Electron Microscopy (TEM)

The cardiac muscle samples were cut into blocks and immersed in 3% glutaraldehyde-paraformaldehyde. The blocks were then dehydrated in a graded series of ethanol and embedded in epoxy resin, with TEM then performed as described previously (Wang et al., 2020a). Semi-thin sections of the tissue samples were stained with methylene blue (Biazik et al., 2015), then adjusted

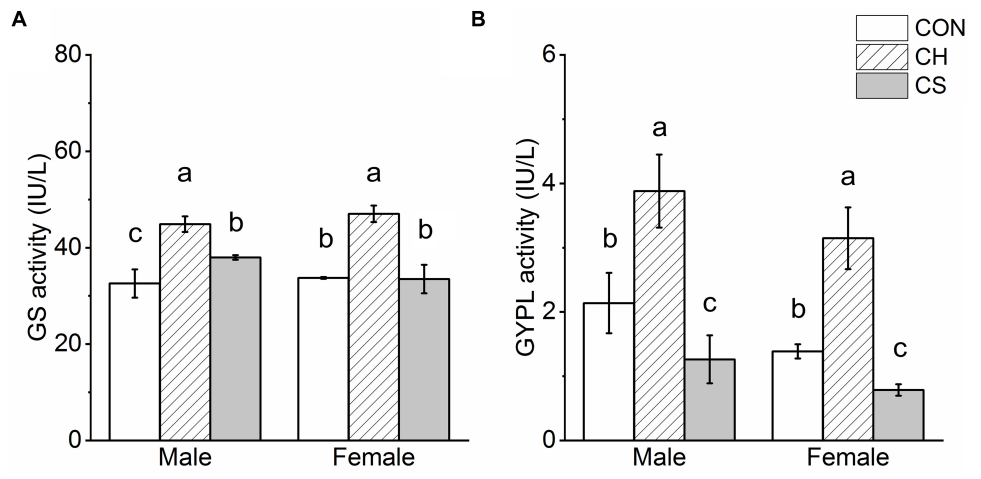

FIGURE 4 | Glycogen synthase (GS) and glycogen phosphorylase (GYPL) activity in cardiac muscle of voles. **(A)** GS activity. **(B)** GYPL activity. Values are mean ± SD. $n = 8$. CON, control group; CH, cool huddling group; CS, cool separated group. Different letters indicate significant differences among temperature treatment groups ($P < 0.05$).

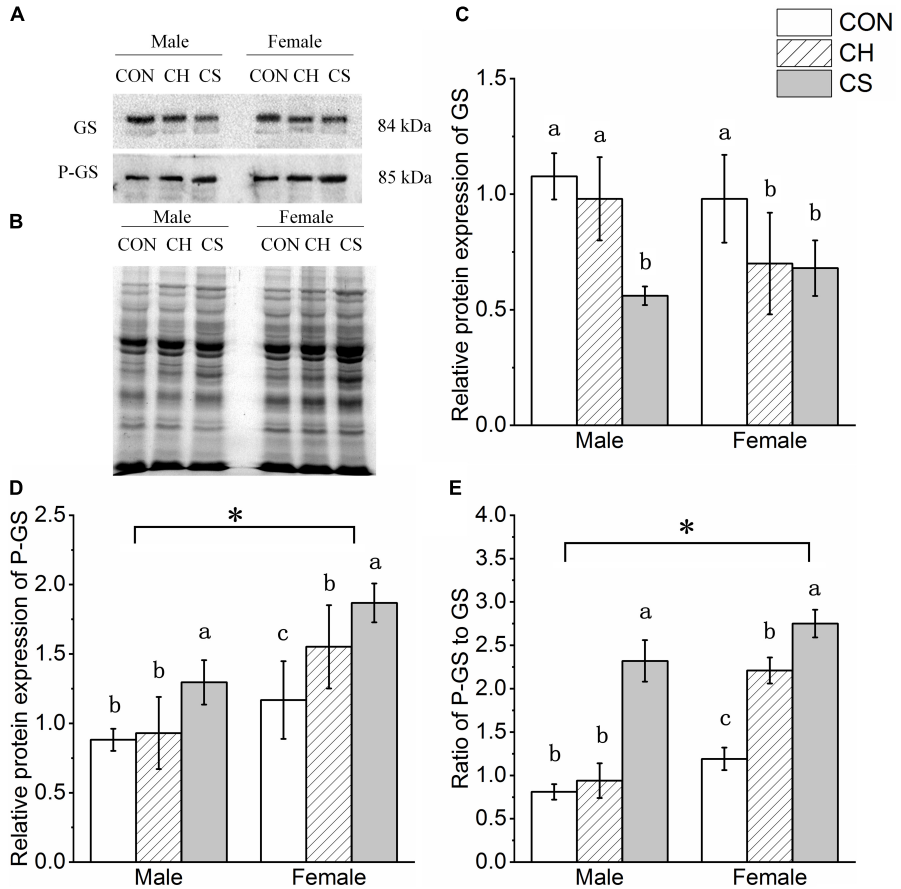

FIGURE 5 | Changes in protein expression levels of glycogen synthesis-related factors in cardiac muscle of Brandt's voles. (A) Representative immunoblots of GS and P-GS in cardiac muscle. **(B)** Representative polyacrylamide gel of total protein. **(C)** Relative protein expression of GS. **(D)** Relative protein expression of P-GS. **(E)** Ratio of P-GS to GS. Values are mean ± SD. $n = 8$. CON, control group; CH, cool huddling group; CS, cool separated group. Different letters identify statistically significant differences among temperature treatment groups ($P < 0.05$). *$P < 0.05$ significant differences between males and females.

under the microscope and sliced with an ultramicrotome (LKB-NOVA, United States). The ultrathin sections were double stained with Reynolds' lead citrate and ethanolic uranyl acetate (Reynolds, 1963) and then examined via TEM (Hitachi, HT7800, Japan). Images were processed with NIH Image-Pro Plus 6.0. Images were analyzed using the measurement tools provided by the software. Glycogenosome densities were determined within a defined region (4 μm^2 area) at a minimum of three locations within an image taken at 25,000 × magnification.

GS and GYPL Activity

Samples stored at −80°C were used to detect GS and GYPL activity. GS activity was determined by measuring the rate of NADH decline at 450 nm using a Glycogen Synthase Assay Kit (20E10Y14, Shanghai Hengyuan Biological Technology Co., Ltd., China) according to the manufacturer's instructions (Ouyang et al., 2018). GYPL activity was determined by measuring the rate of NADPH increase at 450 nm with a Glycogen Phosphorylase Activity Assay Kit (20H10L15, Shanghai Hengyuan Biological Technology Co., Ltd., China) according to the manufacturer's instructions (Song et al., 2018).

Glycogen Quantification

Samples stored at −80°C were used to detect glycogen content. The amount of glycogen in the myocardia from the three groups was determined with a Glycogen Assay Kit (BC0340, Solarbio, Beijing, China). Glycogen levels were normalized by cell protein concentration measured using the BCA assay (Zhao et al., 2017).

Western Blotting

Total protein was extracted from the tissues and solubilized in sample buffer (100 mM Tris, pH 6.8, 5% 2-β-mercaptoethanol, 5% glycerol, 4% SDS, and bromophenol blue), with the extracts of cardiac protein then resolved via SDS-PAGE [10% Laemmli gel with an acrylamide/bisacrylamide ratio of 29:1 and 98% 2,2,2-trichloroethanol (Aladdin, JI522028, China)]. To study protein expression in different tissues, we used total protein content as a reference. After electrophoresis, the gel was irradiated on the UV platform of the electrophoresis gel imaging analysis system (Bio-Rad, California, United States) for 5 min, with the signal then collected. As described previously (Li and Shen, 2013; Posch et al., 2013), the original image captured with no gain was stored. The

fluorescence intensity of each lane (after removal of background fluorescence intensity) was determined with Image-Pro Plus 6.0, which contains an internal reference to correct the fluorescence intensity of the target protein. The proteins were then electrically transferred to polyvinylidene fluoride (PVDF) membranes (0.45 μm pore size) using a Bio-Rad wet transfer apparatus. The blotted membranes were blocked with 5% skimmed milk powder in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) and incubated with rabbit anti-glycogen phosphorylase (1:1,000, #ab198268, Abcam, Cambridge, United Kingdom), rabbit anti-glycogen synthase (1:1,000, #3886, Cell Signaling Technology CST, Danvers, MA, United States), rabbit anti-phospho glycogen synthase (1:1,000, #3891, CST), rabbit anti-glucose transporter type 1 (1:500, #21829, Proteintech, China), rabbit anti-glucose transporter type 2 (1:500, #20436, Proteintech, China), and rabbit anti-glucose transporter type 4 (1:500, #21048, Proteintech, China) in TBS containing 0.1% BSA at 4°C overnight. The membranes were then incubated with IRDye 800 CW goat anti-rabbit secondary antibodies (1:5,000, #31460, Thermo Fisher Scientific, Rockford, IL, United States) for 90 min at room temperature and visualized with an Odyssey scanner (Bio-Rad, CA, United States). Quantification of blots was performed using NIH Image-Pro Plus 6.0.

Statistical Analyses

The normality of data and homogeneity of variance were tested by Shapiro-Wilk and Levene tests, respectively. All data exhibited normal distribution and homogeneous variance. Double-factor variance analysis [two-way analysis of variance (ANOVA)] was used to compare differences between treatment and sex. Results were significant at $P < 0.05$. Data are expressed as mean ± standard deviation (Mean ± SD). All statistical analyses were conducted using SPSS 19.0.

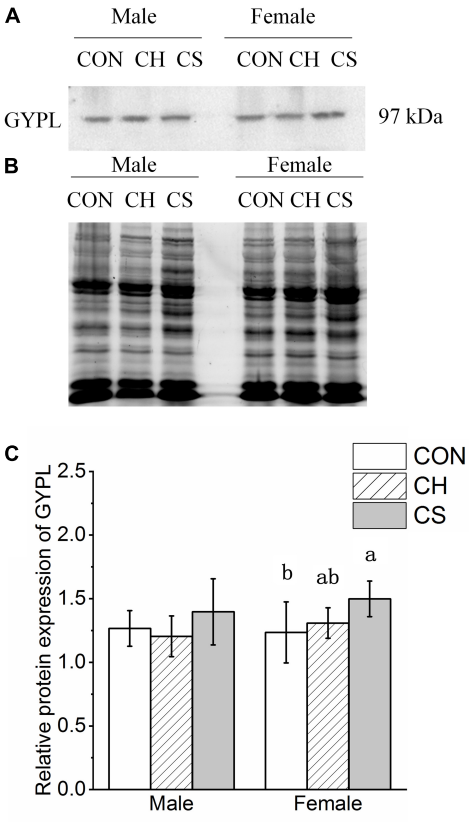

FIGURE 6 | Changes in protein expression levels of glycogen degradation-related factors in cardiac muscle of Brandt's voles. **(A)** Representative immunoblots of GYPL in cardiac muscle. **(B)** Representative polyacrylamide gel of total protein. **(C)** Relative protein expression of GYPL. Values are mean ± SD. $n = 8$. CON, control group; CH, cool huddling group; CS, cool separated group. Different letters identify statistically significant differences among temperature treatment groups ($P < 0.05$).

RESULTS

Ultrastructural Changes in Number of Glycogenosomes

Glycogenosome clusters were observed, with each glycogenosome showing a diameter of ∼30 nm. Most glycogenosomes were distributed between the muscle filaments, with a small number distributed around the mitochondria (**Figure 1**).

Figure 2A shows the distribution of glycogenosomes at low magnification. In the CS group, the number of glycogenosomes was more than triple that in the CON and CH groups ($P < 0.05$). In addition, the number was significantly higher ($P < 0.05$) in females than in males (**Figure 2B**).

Glycogen Quantification

Glycogen quantification showed significant accumulation in the CS group ($P < 0.05$), but no significant differences were observed between the CON and CH groups in either males or females (**Figure 3**).

Changes in GS and GYPL

Results showed that GS activity in the CH group was significantly higher than that in the CON and CS groups ($P < 0.05$), but there were no significant differences between the CON and CS group in females. Furthermore, among the three groups, GYPL activity was highest in the CH group ($P < 0.05$) and lowest in the CS group ($P < 0.05$) (**Figure 4**).

Changes in Protein Expression of Glycogen Synthesis-Related Proteins

The GS and P-GS concentrations were detected by western blot analysis, as shown in **Figure 5**. Representative polyacrylamide gels of total protein are shown in **Figure 5B**.

The relative protein expression levels of GS and P-GS showed different trends among the three treatment groups. Specifically, the protein expression levels of GS in the CS group were lower than the levels in the CH and CON groups, whereas protein expression levels of P-GS in the CH and CS groups

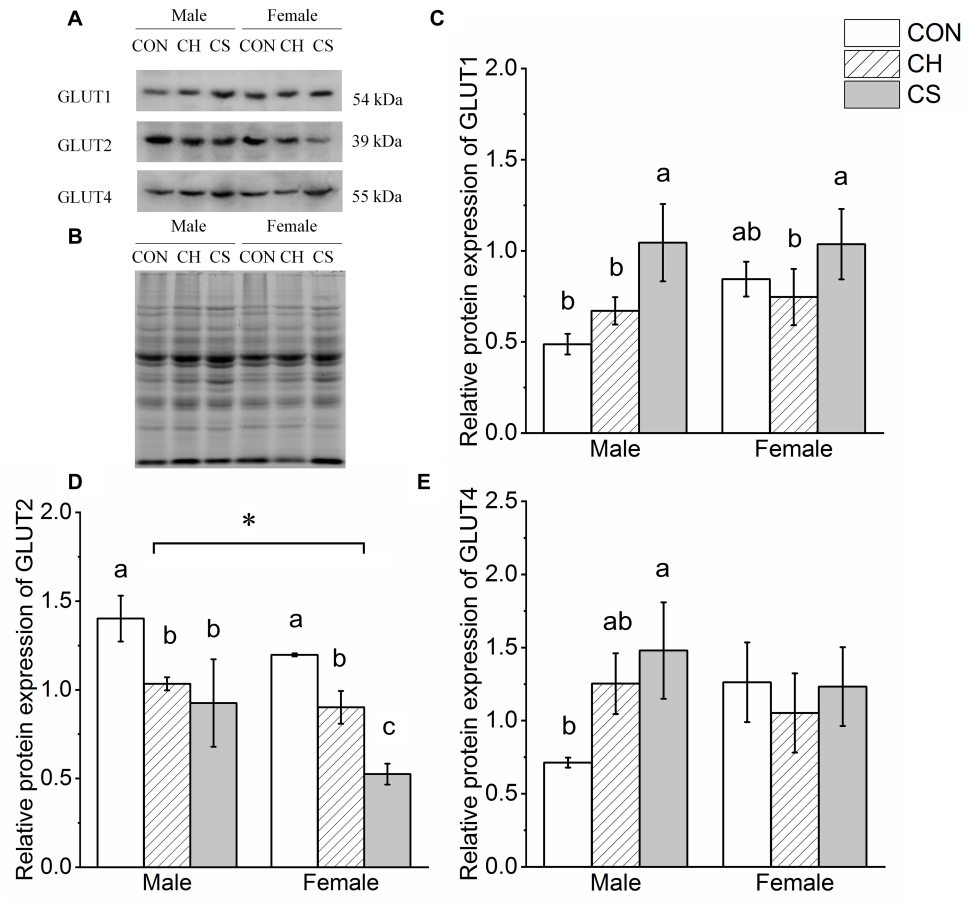

FIGURE 7 | Changes in protein expression levels of glucose transporter proteins in cardiac muscle of Brandt's voles. **(A)** Representative immunoblots of GLUT1, GLUT2, and GLUT4 in cardiac muscle. **(B)** Representative polyacrylamide gel of total protein. **(C)** Relative protein expression of GLUT1. **(D)** Relative protein expression of GLUT2. **(E)** Relative protein expression of GLUT4. Values are mean ± SD. $n = 8$. CON, control group; CH, cool huddling group; CS, cool separated group. Different letters identify statistically significant differences among temperature treatment groups ($P < 0.05$).

were higher than levels in the CON group ($P < 0.05$). Levels of P-GS was higher ($P < 0.05$) in females than in males (**Figures 5C,D**).

The P-GS to GS ratio is one of the most direct indicators of glycogen synthesis. Here, the ratio trend among the three treatment groups was CON < CH < CS ($P < 0.05$). The ratio was also higher ($P < 0.05$) in females than in males (**Figure 5E**).

Changes in Protein Expression of Glycogen Decomposition-Related Proteins

The content of GYPL was detected by western blot analysis, as shown in **Figure 6**. Representative polyacrylamide gels of total protein are shown in **Figure 6B**.

The relative protein expression of GYPL showed a slight change among the three treatment groups. Specifically, levels were higher in CS group females than in CON group females (**Figure 6C**).

Changes in Protein Expression of Glucose Transporter Proteins

The contents of GLUT1, GLUT2, and GLUT4 were detected by western blot analysis, as shown in **Figure 7**. Representative polyacrylamide gels of total protein are shown in **Figure 7B**.

The relative protein expression of GLUT1 increased in the CS group compared with the other groups in both males and females ($P < 0.05$). The relative protein expression of GLUT2 showed the same trend, i.e., CON > CH > CS, but there was a significant difference between males and females. The relative protein expression of GLUT4 in males was markedly higher in the CS group than in the CON group ($P < 0.05$), but there were no differences in females among the three groups.

DISCUSSION

We studied the effects of a cool environment on the number of cardiac glycogenosomes and glycogen content in huddling

Brandt's voles, as well as the underlying mechanism related to the regulation of glycogenosome number. One of the most important findings of this study is the ultrastructural observation of a significant increase in the number of cardiac glycogenosomes in the CS group, as verified by the glycogen content results.

Changes in myocardial glycogen in mammals during long-term cool exposure have not been reported previously, although our results are consistent with those of myocardium under short-term hypothermia and skeletal muscle under long-term hypothermia, as the major types of muscle fibers in ventricles are similar to those in soleus muscle (Schaub et al., 1989). Research on rats has shown that glycogen content in the myocardium is significantly increased after only 4 h of exposure to 15°C (Steffen, 1988). Furthermore, Daurian ground squirrels experience an increase in glycogen concentration in the soleus muscle after 2 months of low temperature exposure in winter (Wang et al., 2019). Excessive glycogen accumulation in the heart can lead to degenerative changes such as arrhythmia, cardiac hypertrophy, and hypotonia (Kanungo et al., 2018). In this study, glycogen content in the myocardium of the CS group was significantly higher than that of the CON group. This indicates that hypothermia may cause significant degenerative damage to the myocardium of small mammals and may involve disrupting the balance between glycogen synthesis and decomposition. In addition, our previous study indicated that ATP synthase activity in the myocardial mitochondria of Brandt's voles under cool conditions is significantly lower than that observed under warm environments, which may lead to a decrease in glucose utilization in the mitochondria (Wang et al., 2020b). Thus, this may be one of the reasons for glycogen accumulation in the CS group.

Here, compared with the CON group, GS activity in the myocardium increased in the CS group males but remained stable in the CS group females, indicating that the level of glycogen synthesis did not decrease. In addition, in the CS group, GYPL activity decreased in the myocardium of both males and females, indicating that glycogen decomposition was weakened. Therefore, the maintenance of glycogen synthesis enzyme and reduction of glycogen degradation enzyme in the CS group may be one of the main reasons for the increase in glycogen content/glycogen particle accumulation in the myocardium. One thing to note is that the expression of GS protein was significantly decreased in the CS group, but its phosphorylation rate, the active state of GS (Greenberg et al., 2006) was significantly increased, which may be a major mechanism related to the unchanged enzyme activity level of GS.

Surprisingly, compared with the CON group, the content of glycogen in the myocardium of the CH group remained unchanged, with the synchronous increase in glycogen synthesis and degradation enzyme likely responsible for the maintenance of glycogen stability. This suggests that the effect of low temperature on glycogen synthesis enzyme can be significantly alleviated by huddling behavior. Here, huddling behavior completely or partially alleviated the increase in glycogen content caused by the decrease in glycogen degradation enzyme in the myocardium of voles following cold environment exposure by increasing glycogen decomposition. Normal glycogen metabolism is the basis of exercise in mammals (Consitt et al., 2019; Moniz et al., 2020). Earlier studies on Brandt's voles showed that activity is higher in huddling groups than separated groups under cool environments (Sukhchuluun et al., 2018). Thus, we speculated that the similar level of glycogen metabolism in the myocardium of the CH group and CON group compared to that in the CS group may be the one of the underlying reasons.

Glycogen synthesis and decomposition also depend on changes in glucose metabolism (Chen and Phelix, 2019). In this study, the protein expression levels of glucose transporters GLUT1 and GLUT4 in the CS group males were significantly higher than in the CON group males, which may contribute to intracellular glucose accumulation and glycogen content increase. In female voles, the protein expression of GLUT1 was significantly higher in the CS group than in the CH group, which may be one of the reasons why glycogen content in the myocardium of the CS group was higher than that of the CH group.

In summary, we explored the regulatory mechanism related to the balance between glycogen synthesis and degradation on the number in myocardial glycogenosomes of huddling and separated Brandt's voles under cool environments. Results showed that a cool environment led to an increase in myocardial glycogen content in voles, which could be alleviated by huddling behavior, and may be a good consequence of the collective overwintering behavior of socialized animals. The activity of glycogen phosphorylase decreased, and the protein expression of GLUT1 and GLUT2 increased in CS group, indicating that the glycogen degradation enzyme decreased and glucose transport increased in the CS group. The activities of glycogen synthase and glycogen phosphorylase increased in the CH group, suggesting that the synthesis and decomposition of glycogen were increased in the CH group. These results indicate that the reduced glycogen degradation enzyme level and enhanced glucose transport may lead to an increase in myocardial glycogen content in the separated voles under cool environment; while the up-regulation of glycogen synthesis and degradation enzyme level maintained myocardial glycogen content in the huddling voles.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

All procedures followed the Laboratory Animal Guidelines for the Ethical Review of Animal Welfare (GB/T 35892-2018) and were approved by the Animal Care and Use Committee of Qufu Normal University (Permit Number: dwsc 2019012).

AUTHOR CONTRIBUTIONS

J-HX and ZW conceived and designed the research, edited the manuscript, approved the final version, and drafted the manuscript. J-HX, ZW, J-JM, C-LW, and W-MH performed the experiments. J-JM and ZW analyzed the data and prepared the figures. J-JM interpreted the experimental results. H-LX, MW, LC, and L-XX provided experimental guidance and suggestions for revision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Vulnerable Plaque Is More Prevalent in Male Individuals at High Risk of Stroke: A Propensity Score-Matched Study

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Objectives: To assess the gender differences in the prevalence of carotid vulnerable plaques in high-risk individuals for stroke in a multicenter, cross-sectional study.

Methods: In the year 2015, 18595 residents who were at the age of 40 or older participated in a face-to-face study in eight communities in southwestern China. Totally 2,644 participants at high risk of stroke were enrolled. Before and after propensity score matching (PSM), the prevalence of carotid plaques and vulnerable plaques were compared between men and women. Multivariate analyses were applied to explore the association between the gender and carotid plaques. Stratified analyses and interaction tests were performed to identify factors that might modify the association between the gender and carotid plaques.

Results: Among 2644 high-risk individuals enrolled, there were 1,202 (45.5%) men and 1442 (54.5%) women. Carotid plaques were detected in 904 (34.2%) participants, while vulnerable plaques were found in 425 (16.1%) participants. Before PSM, carotid plaques were more prevalent in male individuals than the female (36.7% vs. 32.1%, $p = 0.01$), as well as vulnerable plaque (20.0% vs. 12.8%, $p < 0.01$). Men tend to have a higher prevalence of vulnerable plaques in multivariate analyses (adjusted OR 1.70, 95% CI 1.10–2.62, $p = 0.02$). Stratified analyses and interaction tests demonstrated that the association between male sex and vulnerable carotid plaque did not change by age, family history of stroke, histories of chronic disease, smoking status, drinking status, physical activity, and BMI (all p for interaction > 0.05). After PSM, vulnerable plaques were still more prevalent in male individuals than the female (17.03% vs. 12.07%, $p = 0.032$).

Conclusion: Male individuals had a higher risk of vulnerable carotid plaque independent of classical vascular risk factors. Whether there is a gender-specific association between variations in genes related to inflammation, lipid metabolis, and endothelial function and plaque vulnerability needs to be further studied.

Keywords: gender, plaque vulnerability, risk factors, propensity score matching, atherosclerosis

INTRODUCTION

Stroke is one of the leading causes of death and the major cause of adult disability worldwide, especially in China (GBD 2016 Causes of Death Collaborators, 2017; Wu et al., 2019). With the aging of the population, the onging high incidence of risk factors and inadequate management, the burden of stroke is increasing year by year (Wu et al., 2019). Approximately 80% of all strokes are ischemic and carotid artery atherosclerosis accounts for at least 20% of all ischemic strokes (Prasad, 2015; Puig et al., 2020).

Atherosclerosis is an chronic inflammatory disease of the arterial wall, with the characteristics of inflammation, endothelial injury, lipid accumulation, and extensive degradation of extracellular matrix components (Mangge and Almer, 2019; Wijeratne et al., 2020). Carotid atherosclerosis has been identified as a major risk factor of ischemic stroke, cardiovascular diseases, and other vascular events (Rundek et al., 2008; Sillesen et al., 2018; Parish et al., 2019). Ultrasound is a non-invasive and economical diagnostic technique that helps provide valuable information on carotid atherosclerosis such as carotid intima thickness (CIMT) and carotid plaque presence (Park, 2016). Several studies suggest that carotid plaque is more powerful in predicting vascular outcomes, compared with CIMT (Ho, 2016; Nezu and Hosomi, 2020).

Previous epidemiological researches have reported the associations between several classical vascular risk factors (such as age, hypertension, diabetes, dyslipidemia, and current smoking) and carotid plaques (Sturlaugsdottir et al., 2016; Bian et al., 2018; Noflatscher et al., 2019; Santos-Neto et al., 2021). It is noted that the incidence of stroke is higher in male individuals compared with the female age < 75 years (Lloyd-Jones et al., 2010), and gender differences in plaque characteristics might help explain this phenomenon. However, there is scarce information available about the gender differences in the prevalence of carotid plaque in high-risk individuals for stroke. Previous studies which investigated the association between sex and intra-plaque hemorrhage (IPH) mainly focused on patients with moderate or severe carotid stenosis (Ota et al., 2010). Meanwhile, the judgment of IPH in carotid plaque was mainly based on histopathological examination after carotid endarterectomy (CEA) (Hellings et al., 2007). Therefore, we conducted the present study using the data of a multicenter, cross-sectional survey in China to explore the gender differences in the prevalence of carotid plaques among individuals at high risk of stroke.

MATERIALS AND METHODS

Study Design and Participants

The present study was a branch of the China National Stroke Screening Survey (CNSSS) program of the National Health and Family Planning Commission of China (grant No. 2011BAI08B01) (Li et al., 2015; Yi et al., 2020a,b). The CNSSS which aimed to provide stroke prevention policies for the Chinese, is a population-based cross-sectional study with a 2-stage stratified sampling framework (Li et al., 2015; Yi et al., 2020a,b). More details of the CNSSS could be followed at the official website (Stroke Prevention Project Committee, 2018). From May 1, 2015 to Sep 31, 2015, the present study was conducted in eight randomly selected communities of Sichuan province in southwestern China, using a cluster survey method (Yi et al., 2020a,b). This survey was performed among residents aged ≥ 40 and who lived more than 6-month in each community. Ethics Committee of the three participating institutions (People's Hospital of Deyang City, Affiliated Hospital of Southwest Medical University, the Suining Central Hospital) approved our study protocol and written informed consent was obtained from all participants enrolled in this study (Yi et al., 2020a,b).

Data Collection and the Definition of High-Risk Individuals for Stroke

Data were collected via using a standardized structured face-to-face questionnaire by experienced surveyors, including demographic information (age, sex, education level), family history of stroke, behavior factors (smoking, drinking, exercise habits), history of stroke [ischemic stroke or transient ischemic attack (TIA), hemorrhagic stroke], history of chronic diseases (hypertension, dyslipidemia, diabetes mellitus, and atrial fibrillation) (Yi et al., 2020a,b). Body measurements of height, weight, waist circumference, and hip circumference were also measured and recorded in the questionnaire. The eight stroke-related risk factors were assessed, including hypertension, dyslipidemia, diabetes mellitus, atrial fibrillation, current smoking ($\geq$1 cigarette per day), physical inactivity (physical exercise $<$ 3 times per week for $<$ 30 min each time), overweight/obesity [defined as body mass index (BMI) $\geq$ 26 kg/m^2], and a family history of stroke, which has been elaborated upon in our previous study (Yi et al., 2020a,b). Participants who had at least three of the above eight risk factors or had a history of stroke were identified as the high-risk participants for stroke (Wang et al., 2017).

Laboratory examinations such as fasting blood glucose (FBG), hemoglobin A1c, triglycerides, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and homocysteine, electrocardiogram (ECG), and carotid ultrasonography were also obtained from the high-risk participants for stroke (Yi et al., 2020a,b). Detailed methods for data collection have been elaborated upon in our previous studies (Yi et al., 2020a,b).

Carotid Ultrasound Examination

Diagnostic ultrasound (type 512, ACUSON Sequoia Apparatus, 7.5 MHz probe, Berlin, Germany) was performed in participants at high risk of stroke to assessed bilateral common and internal carotid arteries, as well as bifurcations according to standard scanning reading protocols (Rundek et al., 2008; Yi et al., 2020b). Detailed procedures for evaluating the characteristics of carotid plaque have been described in our previous study (Yi et al., 2016, 2017, 2020b). An atherosclerotic plaque was defined as the presence of an endoluminal protrusion $>$ 1.5 mm or a focal thickening at least 50% greater of the CIMT than adjacent arterial wall (Rundek et al., 2008; Yi et al., 2020b).

Based on the plaque echogenicity and surface appearance, carotid plaques were further classified from class I to class IV as uniformly echolucent, predominantly echolucent, predominantly echogenic, and echogenic, respectively (Mathiesen et al., 2001; Yi et al., 2020b). Plaques of class I or II were identified as vulnerable plaques, while plaques of class III or IV were identified as stable plaques (Yi et al., 2016, 2017, 2020b). Carotid plaques were independently classified by ultrasound practitioners who were blinded to baseline information.

Statistical Analyses

Clinical characteristics are presented as means with standard deviations (SDs) for continuous variables and as frequencies with percentages for categorical variables according to different genders. Intergroup differences in categorical variables were calculated for significance using the χ^2-tests or Fisher's exact tests, while intergroup differences in continuous variables were calculated using the Student's *t*-tests or Mann-Whitney *U*-test (Li et al., 2020a).

Univariate analysis comparing factors associated with carotid plaque and vulnerable plaque in high-risk individuals for stroke was performed. Multivariate logistic regression was performed to identify the association between gender and carotid plaque in high-risk individuals in 4 different models. Model 1 was adjusted for age and family history of stroke. Model 2 was adjusted for variables in model 1 + BMI. Model 3 was adjusted for variables in model 1 + BMI + vascular risk factors (history of ischemic stroke or TIA, hypertension, dyslipidemia, diabetes mellitus, smoking status). Model 4 was adjusted for variables in model 1 + BMI + vascular risk factors + laboratory test (Hemoglobin A1c, FBG, Triglycerides, TC, HDL-C, LDL-C). Stratified analyses and interaction tests were conducted according to age, family history of stroke, histories of chronic disease, smoking status, drinking status, physical activity, and BMI, to identify factors that might modify the association between the gender and carotid plaques. The significance of interaction was tested by the log-likelihood ratio test.

We also performed a propensity score matching (PSM) algorithm including baseline characteristics that are assumed to be related to the gender by using a multivariate logistic regression analysis, to calculate the propensity score for each patient. Then participants between different gender groups were matched via using the nearest neighbor approach (caliper 0.2, ratio 1:1) to minimize potential imbalances between the two groups as previously described in detail (Li et al., 2020a). Gender differences in the prevalence of carotid plaques and vulnerable plaques were compared before and after PSM.

The 95% confidence intervals (CI) were calculated to describe the precision of the estimates. Two-sided $P < 0.05$ was considered statistically significant for all results. All statistical analyses were performed using SPSS 21.0 software (IBM, Chicago, IL, United States), statistical software packages R (The R Foundation, version 3.4.3)[1] and EmpowerStats (X&Y Solutions, Inc., Boston, MA, United States)[2], which have been described in our previous studies (Li et al., 2020a,b).

[1]http://www.R-project.org

RESULTS

In the year 2015, 18595 residents aged ≥ 40 participated in the face-to-face survey in eight communities in Sichuan province in southwestern China. Finally, a total of 2644 subjects at high risk of stroke were enrolled, comprising 1,202 men and 1,442 women aged 63.3 ± 9.8 years. A flow diagram of the data preparing and cleaning process in this survey is provided in **Figure 1**.

Gender Differences in the Characteristics of High-Risk Individuals and the Prevalence of Carotid Plaque

Gender differences in the characteristics of high-risk individuals and the prevalence of carotid plaque were exhibited in **Table 1**. Compared with women, men were younger (62.7 ± 10.3 vs. 63.7 ± 9.4 years, $p < 0.01$), had higher educational level ($p < 0.01$), more history of former and current smoking (13.9%, 54.2% vs. 1.7%, 4.4%, respectively, $p < 0.01$) and regular alcohol consumption (18.7% vs. 1.6%, $p < 0.01$), less history of ischemic stroke or transient ischemic stroke (TIA) (14.4% vs. 20.5%, $p < 0.01$), diabetes (28.4% vs. 39.3%, $p < 0.01$), dyslipidemia (67.8% vs. 76.5%, $p < 0.01$) and atrial fibrillation (7.8% vs. 11.0%, $p < 0.01$). Meanwhile, the level of FBG (6.3 ± 2.2 vs. 6.5 ± 2.7 mmol/L, $p = 0.02$), total cholesterol (5.1 ± 1.0 vs. 5.3 ± 1.0 mmol/L, $p < 0.01$), HDL-C (1.5 ± 0.5 vs. 1.7 ± 0.6 mmol/L, $p < 0.01$) and LDL-C (2.9 ± 0.9 vs. 3.1 ± 0.9 mmol/L, $p < 0.01$) were lower in men than in women in the current survey. However, men had a higher level of homocysteine than women (15.5 ± 11.1 vs. 11.9 ± 7.2 mmol/L, $p < 0.01$).

[2]http://www.empowerstats.com

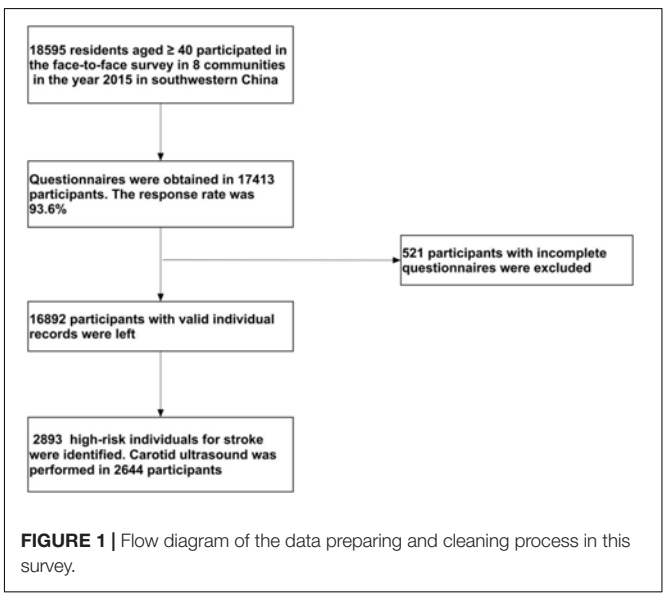

FIGURE 1 | Flow diagram of the data preparing and cleaning process in this survey.

TABLE 1 | Gender differences in the characteristics of individuals at high risk of stroke and the prevalence of carotid plaque.

Variables	Total (*n* = 2,644)	Male (*n* = 1,202)	Female (*n* = 1,442)	*P*-value
Age, year, mean ± SD	63.3 ± 9.8	62.7 ± 10.3	63.7 ± 9.4	<0.01*
Education, n (%)				<0.01‡
Primary school or below	1833 (69.3)	762 (63.4)	1071 (74.3)	–
Junior middle school	658 (24.9)	343 (28.5)	315 (21.8)	–
Senior middle school	120 (4.5)	73 (6.1)	47 (3.3)	–
College or above	33 (1.3)	24 (2.0)	9 (0.6)	–
Family history of stroke, n (%)	474 (17.9)	206 (17.1)	268 (18.6)	0.33‡
Vascular risk factors, n (%)				
Ischemic stroke or TIA	468 (17.7)	173 (14.4)	295 (20.5)	<0.01‡
Hemorrhagic stroke	93 (3.5)	36 (3.0)	57 (4.0)	0.18‡
Hypertension	2122 (80.3)	945 (78.6)	1177 (81.6)	0.05‡
Diabetes mellitus	907 (34.3)	341 (28.4)	566 (39.3)	<0.01‡
Dyslipidemia	1918 (72.5)	815 (67.8)	1103 (76.5)	<0.01‡
Atrial fibrillation	252 (9.5)	94 (7.8)	158 (11.0)	<0.01‡
Smoking status, n (%)				<0.01‡
Never	1736 (65.7)	383 (31.9)	1353 (93.8)	–
Former	192 (7.3)	167 (13.9)	25 (1.7)	–
Current	716 (27.1)	652 (54.2)	64 (4.4)	–
Alcohol consumption, n (%)	248 (9.4)	225 (18.7)	23 (1.6)	<0.01‡
Physical inactivity, n (%)	1698 (64.2)	757 (63.0)	941 (65.3)	0.22‡
Overweight or obesity, n (%)	1647 (62.3)	744 (61.9)	903 (62.6)	0.69‡
BMI, kg/m	26.0 ± 3.6	25.9 ± 3.3	26.1 ± 3.7	0.16*
Waist circumference, cm	88.9 ± 12.0	88.9 ± 10.0	86.4 ± 11.6	<0.01*
Hip circumference, cm	95.2 ± 11.9	95.9 ± 10.5	94.6 ± 13.0	0.16*
Hemoglobin A1c, mmol/L	6.8 ± 1.8	6.9 ± 2.0	6.6 ± 1.6	<0.01*
FBG, mmol/L	6.4 ± 2.5	6.3 ± 2.2	6.5 ± 2.7	0.02*
Total cholesterol, mmol/L	5.2 ± 1.0	5.1 ± 1.0	5.3 ± 1.0	<0.01*
HDL-C, mmol/L	1.6 ± 0.6	1.5 ± 0.5	1.7 ± 0.6	<0.01*
LDL-C, mmol/L	3.0 ± 0.9	2.9 ± 0.9	3.1 ± 0.9	<0.01*
Triglycerides, mmol/L	1.8 ± 1.9	1.8 ± 2.2	1.8 ± 1.6	0.36*
Homocysteine, mmol/L	13.6 ± 9.4	15.5 ± 11.1	11.9 ± 7.2	<0.01*
Total carotid plaque, n (%)	904 (34.2)	441 (36.7)	463 (32.1)	0.01‡
Vulnerable carotid plaque, n (%)	425 (16.1)	240 (20.0)	185 (12.8)	<0.01‡

Data are presented as mean ± SD, median (range), or number (%).
**Student t-test. ‡ $\chi 2$ test.*
BMI, Body mass index; FBG, fasting blood glucose; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

Carotid plaques were found in 904 (34.2%) subjects, and 425 (16.1%) had vulnerable plaques. The total prevalence of carotid plaque was higher in men than in women (36.7% vs. 32.1%, $p = 0.01$), as well as vulnerable plaque (20.0% vs. 12.8%, $p < 0.01$).

Risk Factors Associated With Carotid Plaques in High-Risk Individuals for Stroke

Univariable analysis of risk factors associated with total carotid plaques and vulnerable plaques was presented in **Table 2**. Male sex was associated with both carotid plaque (OR 1.23, 95%CI 1.04–1.44, $p = 0.01$) and vulnerable plaque (OR 1.70, 95%CI 1.37–2.09, $p < 0.01$). Besides, age, history of ischemic stroke/TIA, hypertension, dyslipidemia, former or current smoking, baseline hemoglobin A1c, FBG, TC, and LDL-C were associated with total carotid plaque (all $p < 0.05$). Meanwhile, age, hypertension, former or current smoking, baseline hemoglobin A1c, FBG, triglycerides, TC, and LDL-C were associated with vulnerable plaque (all $p < 0.05$).

TABLE 2 | Univariable analysis for the factors associated with carotid plaques in a population at high risk of stroke.

Variables*	Total carotid plaque		Vulnerable carotid plaque	
	OR (95%CI)	*P*-value	OR (95%CI)	*P*-value
Age, year	1.07 (1.06–1.08)	<0.01	1.05 (1.04–1.06)	<0.01
Male	1.23 (1.04–1.44)	0.01	1.70 (1.37–2.09)	<0.01
Education level				
College or above	Reference	–	Reference	–
Primary school or below	1.22 (0.59–2.52)	0.60	1.56 (0.55–4.48)	0.41
Junior middle school	0.65 (0.31–1.38)	0.26	0.98 (0.33–2.85)	0.96
Senior middle school	0.93 (0.41–1.10)	0.86	1.28 (0.40–4.08)	0.68
Family history of stroke	0.82 (0.66–1.02)	0.07	0.77 (0.57–1.02)	0.07
Vascular risk factors				
Ischemic stroke or TIA	0.71 (0.57–0.88)	<0.01	0.88 (0.67–1.17)	0.39
Hemorrhagic stroke	1.41 (0.93–2.14)	0.11	1.09 (0.63–1.88)	0.76
Hypertension	1.67 (1.35–2.07)	<0.01	1.64 (1.22–2.19)	<0.01
Dyslipidemia	1.22 (1.02–1.47)	0.03	1.20 (0.95–1.53)	0.13
Diabetes mellitus	1.06 (0.90–1.26)	0.50	1.09 (0.88–1.36)	0.42
Atrial fibrillation	1.12 (0.85–1.47)	0.42	1.02 (0.71–1.44)	0.93
Smoking status				
Never	Reference	–	Reference	–
Former	2.04 (1.51–2.75)	<0.01	1.93 (1.34–2.77)	<0.01
Current	1.36 (1.13–1.63)	<0.01	1.70 (1.36–2.14)	<0.01
Alcohol consumption	1.13 (0.86–1.48)	0.38	1.11 (0.78–1.56)	0.57
Physical inactivity	1.00 (0.84–1.18)	0.96	0.92 (0.74–1.14)	0.44
BMI	1.00 (0.98–1.02)	0.99	0.97 (0.95–1.00)	0.09
Waist circumference	1.00 (0.99–1.01)	0.38	1.00 (0.99–1.01)	0.85
Hip circumference	1.00 (0.98–1.01)	0.78	0.99 (0.97–1.01)	0.38
laboratory test				
Hemoglobin A1c	1.08 (1.01–1.16)	0.03	1.09 (1.01–1.18)	0.03
FBG	1.04 (1.01–1.08)	0.01	1.05 (1.01–1.09)	0.01
Triglycerides	1.01 (0.97–1.05)	0.71	1.05 (1.01–1.11)	0.03
TC	1.19 (1.10–1.29)	<0.01	1.21 (1.10–1.33)	<0.01
HDL-C	1.20 (1.04–1.37)	0.01	1.05 (0.88–1.24)	0.59
LDL-C	1.22 (1.11–1.34)	<0.01	1.26 (1.12–1.42)	<0.01
Homocysteine	1.01 (1.00–1.02)	0.06	1.00 (0.99–1.01)	0.72

OR, odds ratio; CI, confidence intervals; BMI, Body mass index; FBG, fasting blood glucose; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

The Association Between Male Sex and Carotid Plaque in High-Risk Individuals for Stroke

As presented in **Table 3**, multivariate logistic regression was conducted to explore the association between male sex and total carotid plaque or vulnerable plaque. After adjusting for age, family history of stroke, and BMI (model 1 or 2), male sex was significantly associated with total carotid plaque ($p < 0.01$) and vulnerable plaque ($p < 0.01$). When vascular risk factors (including a history of ischemic stroke or TIA, hypertension, dyslipidemia, diabetes mellitus, smoking status) and lab tests (including hemoglobin A1c, FBG, triglycerides, TC, HDL-C, LDL-C) were included in the multivariate logistic regression (model 3 or 4), male sex was no longer an independent risk factor for carotid plaque, however, the male was still an independent risk factor for vulnerable plaque (adjusted OR 1.70, 95%CI 1.10–2.62, $p = 0.02$, in model 4) than female.

TABLE 3 | Multiple logistic regression analysis for the association between male sex and carotid plaque in a population at high risk of stroke.

	Total carotid plaque		Vulnerable carotid plaque	
	OR (95%CI)	***P*-value**	**OR (95%CI)**	***P*-value**
Unadjusted	1.23 (1.04–1.44)	0.01	1.70 (1.37–2.09)	<0.01
Model 1	1.33 (1.12–1.58)	<0.01	1.79 (1.45–2.22)	<0.01
Model 2	1.35 (1.14–1.60)	<0.01	1.79 (1.45–2.22)	<0.01
Model 3	1.03 (0.82–1.29)	0.82	1.49 (1.13–1.97)	<0.01
Model 4	1.30 (0.89–1.89)	0.17	1.70 (1.10–2.62)	0.02

Model 1 was adjusted for age and family history of stroke.
Model 2 was adjusted for variables in model 1 + BMI.
Model 3 was adjusted for variables in model 1 + BMI + vascular risk factors (history of ischemic stroke or TIA, hypertension, dyslipidemia, diabetes mellitus, smoking status).
Model 4 was adjusted for variables in model 1 + BMI + vascular risk factors + laboratory test (Hemoglobin A1c, FBG, Triglycerides, TC, HDL-C, LDL-C).
OR, odds ratio; CI, confidence intervals; BMI, Body mass index; FBG, fasting blood glucose; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

Stratified Analyses and Interaction Test of the Association Between Male Sex and Vulnerable Plaque

To further explore the association between male sex and vulnerable carotid plaques, stratified analyses and interaction tests were employed. In **Figure 2**, we found that the association between male sex and vulnerable carotid plaque did not change by age, family history of stroke, histories of chronic disease (ischemic stroke or TIA, hypertension, dyslipidemia, diabetes mellitus), smoking status, drinking status, physical activity and BMI (all p for interaction > 0.05). Male individuals tended to have a stronger association with vulnerable carotid plaque compared with the female, as shown in **Table 3**.

Gender Differences in the Prevalence of Carotid Plaque and Vulnerable Plaque After PSM

After PSM, we identified two subgroups of 928 participants (including 464 men and 464 women) at high risk of stroke that were balanced for all characteristics. Relative multivariate imbalance in terms of the L1 measure was smaller (0.468 vs. 0.733) and no covariate had standardized mean differences > 0.1 after PSM. As shown in **Table 4**, there was no significant difference in the characteristics between different gender groups in the matched dataset (all $p > 0.05$). After PSM, men no longer had more carotid plaque (33.2% vs. 31.9%, $p = 0.67$), however, vulnerable plaques were still more prevalent in male individuals (17.0% vs. 12.1%, $p = 0.03$).

DISCUSSION

Atherosclerosis in the carotid artery can lead to plaque vulnerability, which is one of the main causes of ischemic stroke (Howard et al., 2015; Pelisek et al., 2012). Our present study have identified a high prevalence of total carotid plaque (34.2%) and vulnerable carotid plaque (16.1%) among high-risk participants for stroke in southwestern China and demonstrated that male individuals have a higher risk of vulnerable plaques than the female (adjusted OR 1.70, 95% CI 1.10–2.62), even after propensity score -matched. Moreover, stratified analyses and interaction tests showed that the stronger association between male sex and vulnerable plaque did not change by age, family history of stroke, histories of chronic disease (ischemic stroke or TIA, hypertension, dyslipidemia, diabetes mellitus), smoking status, drinking, physical activity, and BMI, suggesting that male is associated with a higher risk of vulnerable plaque independent of classical vascular risk factors.

It has been demonstrated that age, hypertension, diabetes, high low-density lipoprotein cholesterol levels, and current smoking are traditional cardiovascular risk factors related to the prevalence of carotid plaques (Sturlaugsdottir et al., 2016; Bian et al., 2018; Noflatscher et al., 2019; Santos-Neto et al., 2021). However, there is scarce information regarding the gender differences in the prevalence of carotid plaques in participants at high risk of stroke, especially vulnerable plaque. It is known that IPH is one of the major characteristics of vulnerable plaque, several researchers have investigated the association between sex and IPH in the carotid artery (Hellings et al., 2007; Ota et al., 2010; Vrijenhoek et al., 2013). Observational research based on histological analysis of CEA specimens found that female individuals tend to have a more stable, less inflammatory carotid plaques compared with the male, independent of clinical manifestation and cardiovascular risk factors (Hellings et al., 2007). Similarly, a cohort study conducted in patients who had undergone CEA suggested that carotid plaques obtained from male individuals had a higher prevalence of IPH compared with the female (Vrijenhoek et al., 2013). Another study enrolled patients with asymptomatic moderate or severe carotid stenosis, and suggested that men had more high-risk plaques compared with women after justing for potential

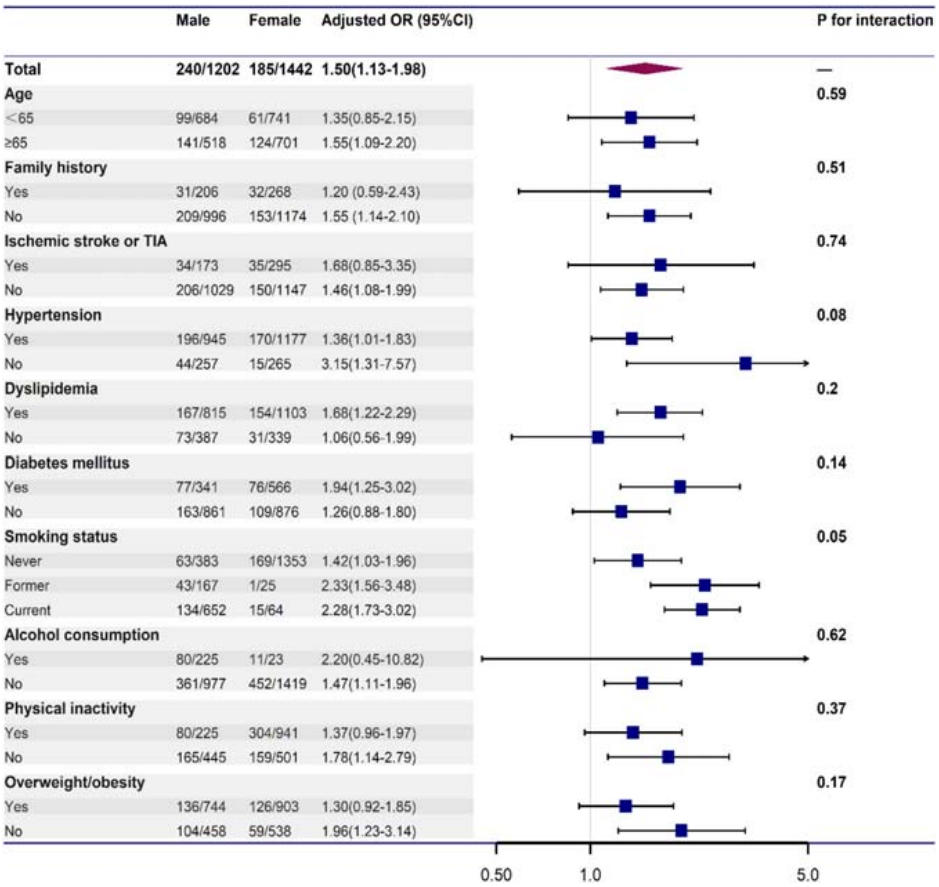

FIGURE 2 | Stratified logistic regression analysis to identify variables that modify the association between male and vulnerable carotid plaque. Each stratification was adjusted for age, family history of stroke, histories of chronic disease (ischemic stroke or TIA, hypertension, dyslipidemia, diabetes mellitus), smoking status, regular alcohol consumption, physical inactivity, and BMI, except for the stratification factor itself.

confounders (Ota et al., 2010). Our study demonstrated that male individuals had a higher risk of vulnerable carotid plaques than the females, which is similar to the results of previous studies (Hellings et al., 2007; Ota et al., 2010; Vrijenhoek et al., 2013). The difference between our study and previous studies is that the current study was a population-based study conducted in high-risk individuals for stroke, which is different from previous studies that mainly focused on patients with moderate or severe carotid stenosis, even posttreatment of CEA.

The underlying pathophysiologic mechanisms that explain these gender differences of the prevalence of vulnerable carotid plaque are poorly understood. There are several possible reasons for this. First, although men do not experience a rapid decline in endogenous sex hormone production, an age-related decrease in the levels of endogenous sex hormone especially testosterone might have an important effect on the progression of atherosclerosis. It has been demonstrated that low levels of free testosterone are associated with the progression of carotid atherosclerosis in elderly men independently of classical vascular risk factors (Muller et al., 2004; Svartberg et al., 2006; Soisson et al., 2012). Second, there are differences in the protective effect of estrogen on atherosclerosis between the two genders (Yahagi et al., 2015). Estrogen might play a direct effect on matrix metalloproteinase production contributing to the attenuation of atherosclerotic disease in females (Yahagi et al., 2015). A recently published observational study found that men have more age-specific carotid IPH in magnetic resonance imaging compared with women. However, among post-menopausal women, the risk of carotid IPH becomes closer to that of men with increasing age (Singh et al., 2017). It has been found that men with the common genetic variation in estrogen receptor alpha have three times higher risk of myocardial infarction as compared to those without variant (Shearman et al., 2003), which indicates that genetic factors might play an essential role in the gender differences of atherosclerosis. A previous study found that only 19.5% of the carotid plaque burden could be explained by traditional and less traditional vascular risk factors, also suggesting that genetic and environmental factors might play a major role in the determination of atherosclerosis (Kuo et al., 2012). Until recently, variation in genes related to inflammation, endothelial function, and lipid metabolism are thought to be linked to carotid plaque burden (Gardener et al., 2011; Wang et al., 2011; Stroke Prevention Project Committee, 2018). Whether there is a gender-specific association between variations in genes related to inflammation, endothelial function, and lipid metabolism and plaque vulnerability has not been adequately studied.

TABLE 4 | Gender differences in the characteristics of individuals at high risk of stroke and the prevalence of carotid plaque after PSM.

Variables	Total (*n* = 928)	Male (*n* = 464)	Female (*n* = 464)	*P*-value
Age, year, mean ± SD	63.1 ± 10.0	63.2 ± 10.4	63.0 ± 9.7	0.77*
Education, n (%)				0.215‡
Primary school or below	620 (66.8)	297 (64.0)	323 (69.6)	–
Junior middle school	256 (27.6)	136 (29.3)	120 (25.9)	–
Senior middle school	42 (4.5)	26 (5.6)	16 (3.5)	–
College or above	10 (1.1)	5 (1.1)	5 (1.1)	–
Family history of stroke, n (%)	179 (19.3)	85 (18.3)	94 (20.3)	0.45‡
Vascular risk factors, n (%)				
Ischemic stroke	151 (16.3)	72 (15.5)	79 (17.0)	0.53‡
Hemorrhagic stroke	33 (3.6)	19 (4.1)	14 (3.0)	0.38‡
Hypertension	763 (82.2)	383 (82.5)	380 (81.9)	0.80‡
Diabetes mellitus	328 (35.3)	158 (34.1)	170 (36.6)	0.41‡
Dyslipidemia	651 (70.2)	332 (71.6)	319 (68.8)	0.35‡
Atrial fibrillation	89 (9.6)	47 (10.1)	42 (9.1)	0.58‡
Smoking status, n (%)				0.28‡
Never	750 (80.8)	375 (80.8)	375 (80.8)	
Former	41 (4.4)	16 (3.5)	25 (5.4)	
Current	137 (14.8)	73 (15.7)	64 (13.8)	
Alcohol consumption, n (%)	51 (5.5)	29 (6.3)	22 (4.7)	0.31‡
Physical inactivity, n (%)	587 (63.3)	285 (61.4)	302 (65.1)	0.25‡
Overweight or obesity, n (%)	595 (64.1)	301 (64.9)	294 (63.4)	0.63‡
BMI, kg/m2	26.5 ± 3.5	26.2 ± 3.3	26.3 ± 3.8	0.53*
Waist circumference, cm	89.2 ± 8.9	87.0 ± 11.2	86.6 ± 10.6	0.61*
Hip circumference, cm	95.2 ± 11.0	95.9 ± 9.8	94.5 ± 12.2	0.34*
FBG, mmol/L	6.6 ± 2.8	6.5 ± 2.7	6.7 ± 2.9	0.28*
Total cholesterol, mmol/L	5.2 ± 1.1	5.2 ± 1.1	5.3 ± 1.1	0.30*
HDL-C, mmol/L	1.7 ± 0.7	1.7 ± 0.7	1.7 ± 0.7	0.63*
LDL-C, mmol/L	3.0 ± 0.8	2.9 ± 0.9	3.0 ± 0.8	0.80*
Triglycerides, mmol/L	1.7 ± 1.6	1.7 ± 1.6	1.7 ± 1.8	0.90*
Homocysteine, mmol/L	7.8 ± 1.3	7.8 ± 1.2	7.8 ± 1.3	0.93*
Total carotid plaque, n (%)	302 (32.5)	154 (33.2)	148 (31.9)	0.67‡
Carotid vulnerable plaque, n (%)	135 (14.6)	79 (17.0)	56 (12.1)	0.03‡

Data are presented as mean ± SD, median (range), or number (%).
**Student t-test. ‡χ^2-test.*
BMI, Body mass index; FBG, fasting blood glucose; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

Limitations

The results of the present study should be interpreted with caution given its limitations. First, although a standardized structured face-to-face questionnaire was used by experienced surveyors to collect data including demographic characteristics, behavior factors, family history of stroke, history of stroke and chronic disease, and physical examination, the application of the self-reported questionnaire might also be associated with recall bias and make the answers unreliable. Second, even though we conduct a multicenter population-based study with a large number of subjects recruited and the large number of variables collected, we only screened residents ages ≥ 40 years and we did not compare the gender differences of carotid plaque in residents who were not identified as the high-risk individuals for stroke, therefore, our results might not represent the whole population. Third, carotid plaque and plaque vulnerability were evaluated by carotid ultrasound but not high-resolution magnetic resonance imaging, which could provide more information including plaque composition and morphology. Besides, the data collection was done many years ago and this is unlikely to provide an updated picture of the situation. Furthermore, we did not explore the effect of antiplatelet drugs or statins on plaque vulnerability in our study due to a lack of data. Finally, limited to the study protocol of the CNSSS program, we could not provide information related to inflammatory markers such as the level of C-reactive protein or other acute-phase protein, and further studies are needed to explore this issue.

CONCLUSION

Despite the above limitations, this multicenter, cross-sectional study provides clear evidence that male individuals had a higher risk of vulnerable carotid plaque independent of classical vascular risk factors, genetic factors might play a major role in the gender differences in the progression of atherosclerosis. Whether there is a gender-specific association between variations in genes involved in inflammation, endothelial function, and lipid metabolism and plaque vulnerability needs to be further studied.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the corresponding author on reasonable request.

ETHICS STATEMENT

Our study protocol was approved by the ethics committee of three participating hospitals (the People's Hospital of Deyang City, the Affiliated Hospital of Southwest Medical University, and the Suining Central Hospital). Informed consents were obtained from all participants during recruitment. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JL and LG collected, analyzed, and interpreted the data, as well as drafted the manuscript. PZ, YL,

and JZ participated in study conception and design, data interpretation, and revised the manuscript. XY and CW contributed substantially to study design and supervision, data interpretation, and manuscript writing. All authors critically revised the manuscript for important intellectual content and approved the final manuscript.

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Sex and Response to Cardioprotective Conditioning Maneuvers

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Ischemic heart disease (IHD) is a multifactorial pathological condition strictly related to genetic, dietary, and lifestyle factors. Its morbidity and mortality rate represent one of the most important pathological issues that today involve younger people in a stronger way than in the past. IHD clinical outcomes are difficult to treat and have a high economic impact on health care. So prevention of this pathological condition through cardioprotective maneuvers represents the first line of intervention, as already underlined by several animal and human studies. Even if the time of intervention is important to prevent severe outcomes, many studies highlight that sex-dependent responses are crucial for the result of cardioprotective procedures. In this scenario sexual hormones have revealed an important role in cardioprotective approach, as women seem to be more protected toward cardiac insults when compared to male counterparts. The aim of this mini review is to show the molecular pathways involved in cardioprotective protocols and to elucidate how sexual hormones can contribute in ameliorating or worsening the physiological responses to IHD.

Keywords: cardioprotection, ischemic heart disease, estrogen, sex, conditioning, gender, reperfusion injury

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INTRODUCTION

Ischemic heart disease (IHD) is a pathological condition characterized by reduced or absent blood flow in coronary arteries due to total or partial occlusion of these vessels by atherosclerotic plaque or blood clots formation. This condition causes improper supply of oxygen and nutrients to myocardial tissue, and, depending on ischemia duration, myocardial cell death can occur. Among all cardiovascular diseases (CVD), IHD is the leading single cause of death in Europe: IHD mortality represents 19% of deaths among men and 20% of deaths among women (Kuznetsova, 2018). Emerging evidences support differences in risk factors, symptoms, and outcomes of IHD between sexes in an age-related trend (Pagliaro et al., 2020). Data from the CVD statistics point out that IHD is the leading cause of death in men (16%) and women (11%) under 75, while it is the single cause of premature mortality in men (16%) under 65 and the second in women compared for the same age ranges (Wilkins et al., 2017). Ischemia can also be induced during cardiac surgery where controlled periods of cardiac arrest allow and optimize critical interventions.

Important cellular modifications during prolonged ischemia are linked to mitochondrial dysfunction and reduced ATP availability and are strictly related to intracellular Ca^{2+} impairment as already reviewed elsewhere (Garcia-Dorado et al., 2012). Furthermore, it is now well established

that reperfusion, that is, the restoration of blood flow, causes further damage of cardiac tissue, maintaining intracellular Ca^{2+} overload that exacerbates the harmful effects induced by ischemia and activates calpain-mediated proteolysis, leading to the condition commonly designated as "reperfusion injury" (Garcia-Dorado et al., 2012; Inserte et al., 2012; Kalogeris et al., 2012).

According to the phenomena described, it is accepted nowadays that ischemia reperfusion injury (IRI) is characterized by detrimental effects of both ischemia and reperfusion. From a clinical perspective, even if restoration of blood flow could be dangerous, it represents the only possible intervention to have better survival chance when coronary occlusion occurs, and researches are moving toward new insights to reduce IRI and improve cardiac outcomes after prolonged ischemia.

Experimental and clinical studies have underlined sex differences in response to IRI, with women showing most detrimental outcomes, probably due to improper diagnosis and poorer prevention procedures than male counterparts (Garcia et al., 2016). IHD shows indeed different onset and clinical pictures in the two sexes, and the underestimated risk for female patients could represent the cause of poorer outcomes (Maas and Appelman, 2010). Moreover, studies are addressed to the development of therapeutic approaches in order to reduce or prevent detrimental effects of IRI in both pathological condition and cardiac surgery (Penna et al., 2015). So in the following paragraphs, we will discuss some cardioprotective maneuvers, in particular pre-conditioning (PreC), post-conditioning (PostC), and remote conditioning, that will be presented with a focus on sexual differences to these interventions, emphasizing the need for a sex-dependent approach in cardioprotection.

SEX DIFFERENCES IN PHYSIOLOGICAL CARDIOPROTECTION

Nowadays it is largely accepted that there are different IRI manifestations in male and female subjects, among all ages and outcome of cardiac injury. Differences studied and results obtained suggest a possible role of sexual hormones in several animal models.

Despite the traditional dualism of estrogens and androgens in conditioning the cardioprotective responses, mainly highlighted by experiments from animal models, human studies and clinical data show a more complex scenario. In this section, cardioprotective pathways activated by both estrogens and androgens are presented, with the aim to underline the weakness of sex-adapted cardioprotective strategies only focused on these mechanisms.

Estrogen binds to different receptors: estrogen receptor-α (ER-α), estrogen receptor-β (ER-β), or G-protein coupled estrogen receptor (GPR30 or GPER). Even if ER-β has a strategical role in cardioprotection, the involvement of ER-α against IRI effects is still controversial; nevertheless, consistent data suggest that estrogen-mediated protective response may rely on both ERs (Murphy and Steenbergen, 2007b; Deschamps et al., 2010). Several pathways activated by this hormone can be seen as the opposite to those activated during ischemia or reperfusion. In fact, estrogen is involved in the regulation of ions transporters and exchangers: it induces S-nitrosylation of L-type Ca^{2+} channels with a reduction of Ca^{2+} loading, and it regulates Ca^{2+} uptake in mitochondria through the extracellular signal-regulated kinases (ERK1/2) (Iorga et al., 2017). Despite reactive oxygen species (ROS) increase being directly related to ischemia duration, it has been demonstrated that estrogen increases mitochondrial biogenesis and reduces ROS production in these cellular compartments. The hormone is also involved in the upregulation of nitric oxide synthases (NOS), through PI3-K pathway, with the consequent rise in NO production that has a primary role in activating protein kinase G (PKG) that enhances K_{ATP} channels activity and inhibits mPTP opening. Evidences show that mitochondrial preservation induced by estrogen could also be mediated by STAT3 activation through tumor necrosis factor receptor 2 (TNFR2) (Wang et al., 2008). Furthermore, estradiol decreases connexin-43 (Cx-43) dephosphorylation, which has been shown to be cardioprotective toward IRI (Murphy and Steenbergen, 2007a,b; Wang et al., 2020).

Cardioprotection in female hearts involves also endogenous antioxidant systems: catalase, superoxide dismutase (SOD), glutathione (GSH), and GSH peroxidase (GPx) are highly expressed if compared to male counterparts (Casin and Kohr, 2020). Moreover, female cardiomyocytes show high ascorbate redox homeostasis and enhanced nitrate-to-nitrite conversion that elevates NO production (Lim et al., 2009).

Other animal studies centered their work in the evaluation of the effect of testosterone in cardioprotection, and like estrogen, androgens can activate both genomic and non-genomic responses by binding to androgen receptors (AR) (Lucas-Herald et al., 2017).

There are several factors through which androgens may have a role in cardioprotection (Bell et al., 2011; Pongkan et al., 2015, 2016; Lucas-Herald et al., 2017). In fact, testosterone is involved in physiological mitochondrial ROS generation (Pagliaro and Penna, 2015); it is also able to activate the PI3-K pathway increasing endothelial NOS (eNOS) activity and NO production. Testosterone is involved in the upregulation of sarcoplasmic reticulum Ca^{2+} release channels (SERCA) and activation of L-type calcium channels, and it has been observed that in severe ischemic condition it may contribute to Ca^{2+} overload suggesting that specific context may modulate the response to this sexual hormone (Murphy and Steenbergen, 2007a; Wang et al., 2008). The positive or negative roles of androgens in cardiac tissue are under investigation, but it is already known that testosterone can be converted to estrogen by aromatase, and through this modification it can activate cardioprotective effects induced by the other sexual hormone.

So reducing the cardioprotective difference between the two sexes relying only on sexual hormones could be limiting in the management of IRI, and it is not supported by pharmacological interventions, with synthetic estrogens that demonstrated detrimental effects in postmenopausal women after IRI (Hulley et al., 1998; Anderson et al., 2004; Sivasinprasasn et al., 2016). Furthermore, new insights underline the involvement of several

determinants in male and female manifestations of IRI, among which are anatomical, physiological, and genetic factors (Luczak and Leinwand, 2009; Regitz-Zagrosek and Kararigas, 2017; Stone et al., 2019; Litviňuková et al., 2020).

SEX AND CARDIOPROTECTIVE MANEUVERS

Regarding the deleterious effects induced by IRI, future challenges for clinical procedures are addressed to the reduction of reversible or not reversible injury of cardiac tissue and the enhancement of cardioprotection. Conditioning maneuvers figure as intriguing protocols that revealed successful results in animal models of IRI, but their potential application to humans seems to underline some difficulties. In particular, PreC is not applicable in pathological settings because of ischemic unpredictability, and only a few clinical trials develop this protocol in cardiac surgery, while PostC seems to be more applicable after ischemic injury even if the window of protection occurs only a few minutes after reperfusion (Kloner and Rezkalla, 2006; Vinten-Johansen et al., 2007). Remote conditioning probably is the most plausible procedure to induce cardioprotection in clinical settings, and several clinical trials are developed to study the advantageous use of this maneuver (Candilio et al., 2011; Pedersen et al., 2018; England et al., 2019). There are plenty of data focusing on the cardioprotective effects of conditioning maneuvers on animal models, and only a few clinical trials developed in humans show contrasting results underlining the difficult translatability of these protocols to clinics (Kloner and Rezkalla, 2006; Peart and Headrick, 2009; Heusch, 2013). The following paragraphs show different sex responses to conditioning procedures in animal models, focusing on possible application to humans. Human studies and most relevant clinical trials based on the effect of conditioning are summarized in **Table 1**.

PreC

Among cardioprotective maneuvers PreC, is a possible therapeutic approach to limit the damaging effect of IRI. PreC consists of brief periods of ischemia and reperfusion (I/R) before the infarcting ischemia; it could be performed through mechanical or pharmacological stimulation or with exercise (Penna et al., 2020). According to its temporal application PreC could be used prior to cardiac surgery interventions, like coronary artery bypass graft surgery (CABG), but it is still difficult to perform PreC in the prevention of other unpredictable cardiac outcomes that involve IRI. Cardioprotection induced by PreC has been demonstrated to involve different molecular pathways that contribute to mitochondrial preservation through the inhibition of mPTP opening; among them are cGMP/PKG pathway that is involved in the production of NO through Akt/eNOS activation, the reperfusion injury savage kinase (RISK) pathway that activates ERK1/2 and GSK3β, and the survivor activating factor enhancement (SAFE) pathway that activates STAT3 responses (Heusch, 2013; Penna et al., 2015).

Given the effect of PreC, and the implications of sex hormones in cardioprotection, it could be interesting to evaluate if there are different responses to PreC in male and female subjects.

In order to assess any difference between sexes in PreC followed by ischemia, it has been evidenced in animal models that not only sex but also age influences the resulting cardioprotection: in fact, young female animals show no response to PreC presumably due to incomplete sexual maturation, while male subjects show cardioprotection after PreC at any age (Turcato et al., 2006).

Furthermore, several studies underline positive effects of PreC before IRI in males but no effects or worst injury in female subjects. Some results suggest that the female heart is physiologically protected by estrogen, and thus the effect of PreC can be insufficient to overcome that of the sexual hormone, while male subjects benefit at all ages from the treatment with PreC. To support this hypothesis, some groups underline the role of different cardioprotective mediators in PreC and their physiological expression in the two sexes: K_{ATP} channels and heat shock proteins (HSP) that are constitutively more activated in females (Ranki et al., 2002; Deschamps et al., 2010), the enzymatic or non-enzymatic capability to control increasing ROS that is more evident in female hearts (Casin and Kohr, 2020), and PI3-K/NOS pathway activation through Notch1 and GPR30 that is more expressed in female hearts (Rocca et al., 2018). On the other hand, a study by Lieder and collaborators underlines no sex differences after PreC treatment of rat hearts showing no sex-dependent differences in infarct size; the authors discuss their results underlining that diverging data in this field could be caused by animal models or different experimental protocols (Lieder et al., 2019). In this scenario, it is highly supported that female cardioprotection induced by PreC can be reached only if cardiac insults overcome a stress threshold that makes the existing estrogen protection insufficient (Song et al., 2003; Pitcher et al., 2005); lack of clinical trials or human studies that show different responses in the two sexes compels this consideration only to a theoretical field.

PostC

Another cardioprotective intervention that can be used to reduce IRI is PostC: brief intermittent cycles of I/R mechanically or pharmacologically induced in the onset of reperfusion. PostC activates different intracellular responses; the most studied are the cGMP/PKG pathway, the RISK pathway, and the SAFE pathway that all contribute to mitochondrial preservation and limit cardiac damage (Heusch, 2013). Emerging clinical studies are addressed to translate animal model results to humans, also because PostC has a great clinical potential and its application could be promising in cardioprotective strategies (Vinten-Johansen et al., 2007). As seen in PreC, it could be reasonable to think that differences between sexes can also be found in PostC responses (Skyschally et al., 2009).

It has been observed that after PostC only males show improved post-ischemic recovery of function, and protective effects of PostC in male subjects involve different factors, such as reduced superoxide production, increased MnSOD expression, reduced Bax/Bcl-2 ratio, and reduced caspase-3

TABLE 1 | Human studies and most relevant clinical trials based on the effect of conditioning maneuvers and their effect in the two sexes.

Intervention	Cardioprotective maneuver	Effect of the treatment	Sex differences	References
Coronary angioplasty for acute myocardial infarction	PostC	Reduction in infarct size and attenuation of no reflow	No sex differences studied	Staat et al., 2005
Primary percutaneous coronary intervention in STEMI	Combination of RIC and PostC	Improvement of myocardial salvage index. No differences in infarct size and microvascular occlusion	No sex differences studied	Eitel et al., 2015
Primary percutaneous coronary intervention in STEMI	RIC	Improvement of myocardial salvage index	No significant sex differences	Sloth et al., 2015
Elective coronary bypass grafting	RIC	No differences in biomarkers release	No interaction between cardioprotection and sex	Kleinbongard et al., 2016
Primary percutaneous coronary intervention in STEMI	RIPC	Reduction in enzymatic infarct size. Improvement of T2-weighted edema volume. ST-segment resolution > 50%	No sex differences studied	Crimi et al., 2013

STEMI, ST-segment elevation myocardial infarction; PostC, post-conditioning; RIC, remote ischemic conditioning; RIPC, remote ischemic post-conditioning.

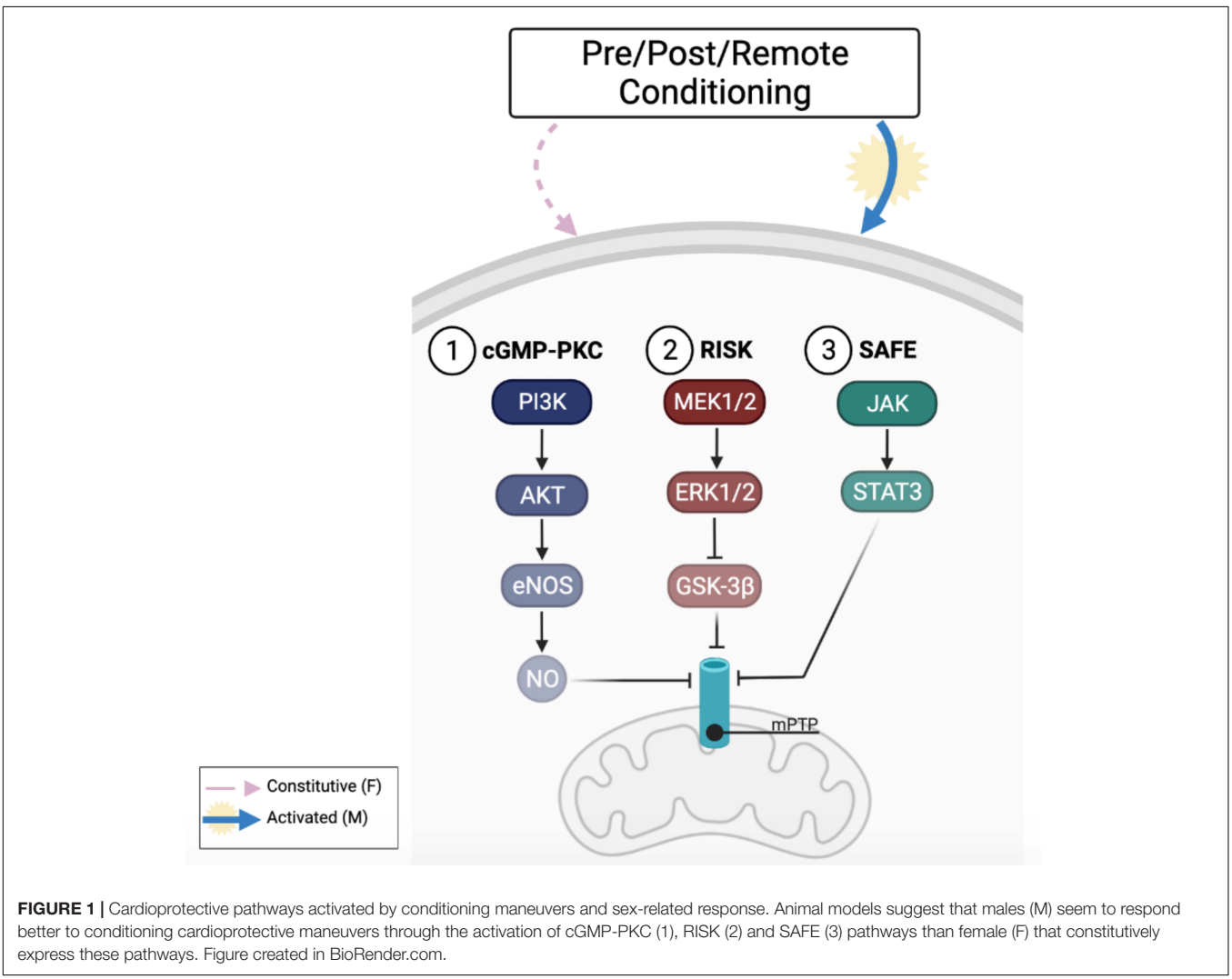

FIGURE 1 | Cardioprotective pathways activated by conditioning maneuvers and sex-related response. Animal models suggest that males (M) seem to respond better to conditioning cardioprotective maneuvers through the activation of cGMP-PKC (1), RISK (2) and SAFE (3) pathways than female (F) that constitutively express these pathways. Figure created in BioRender.com.

activation (Ciocci Pardo et al., 2018). Furthermore, after PostC male hearts also show higher expression in p-Akt, p-GSK3β and p-PKCε, while female expression of these intracellular mediators has no changes after the treatment (Ciocci Pardo et al., 2018). Moreover, Inserte and collaborators show that in male Sprague–Dawley rat hearts, PostC activates the cGMP/PKG pathway that is

involved in cardioprotection through the delaying normalization of intracellular pH (Inserte et al., 2011). Studies on animal models show that PostC maneuvers have effects only in male hearts; lack of data in female animal models make it difficult to define clear differences between sexes.

Regarding all the molecular responses to PostC in males hearts, it could be speculated that PostC response is also strictly related to sex hormones showing that probably it activates pathways that are already highly expressed in females due to estrogen stimulation; so to have PostC cardioprotective effect, it could be reasonable that female hearts have to be exposed to higher injury (Crisostomo et al., 2006; Penna et al., 2009). Some human clinical trials and meta-data analysis underline no beneficial effect of PostC application, but the different sex response was not considered by these studies (Eitel et al., 2015; Xing et al., 2019), while other works show a positive effect of PostC in reducing infarct size, but no sex differences were outlined (Staat et al., 2005). Furthermore, some evidences also underline that the hearts of women treated with PostC showed worse outcomes compared to untreated women, pointing out the need for deeper investigations on the possible application of PostC in female patients (Shin et al., 2019).

Remote Conditioning

Remote ischemic conditioning (RIC) is considered among cardioprotective maneuvers; it consists of brief cycles of ischemia and reperfusion in a peripheral organ or tissue (even in arms or legs), remote from the heart (Przyklenk et al., 1993). It can be induced before (remote PreC), during (remote per-conditioning), or after (remote PostC) an index ischemia (Penna et al., 2015). It is a non-invasive and low-cost procedure that can be performed through an inflating/deflating pneumatic cuff to induce 5 min cycles of ischemia/reperfusion favoring the protection induced by RIC (Hausenloy et al., 2020). Clinical benefits of this procedure are still debated (Hausenloy et al., 2019, 2020), but recent findings point out a possible role of humoral factors released after RIC that have an age- and sex-dependent protective role (Heinen et al., 2018). In particular, Heinen et al. (2018) point out a significant protective role of humoral factors derived from young males exposed to RIC in reducing infarct size, and this is probably due to the phosphorylation of GSK3β that is involved in the inhibition of mPTP through the RISK pathway. Furthermore, a recent work by Lieder et al., reported no differences in cardioprotection in a specific RIC model (Lieder et al., 2019). Clinical data suggest controversial results on the efficacy of RIC, some of them showing no cardioprotection (García Del Blanco et al., 2021), while others underlining cardioprotective effects of RIC with no sex differences (Crimi et al., 2013; Eitel et al., 2015; Sloth et al., 2015; Kleinbongard et al., 2016). Insufficient data in animal and clinical studies point out differences between sexes in cardioprotective effects of RIC, and some authors suggest estrogens as possible confounding factors that make difficult the interpretation of limited data regarding the role of RIC in female subjects (Brevoord et al., 2012; Lieder et al., 2019; Shaban and Leira, 2019).

CONCLUSION

In this brief report, we have outlined different responses in female and male hearts to IRI, and in particular we focused on a primary role of estrogens in cardioprotection. Some of the most studied cardioprotective maneuvers, in order to reduce IRI, have been described with a particular focus on females and males' different responses. The overview outlined here shows that differences between sexes in cardioprotective interventions could be linked, but not exclusively, to the physiological role of sexual hormones that change throughout the lifespan highlighting a complex relationship toward age and sex in response to cardioprotective maneuvers. In conclusion, sex and age differences have to be considered in cardioprotection in order to optimize the clinical application of these procedures. As **Figure 1** shows, we have focused on intrinsic cardioprotective mechanisms that can be elicited by conditioning maneuvers as in our opinion it is important to understand the sex-related differences in these mechanisms before moving on to testing pharmaceutical approaches. Furthermore, a deeper knowledge of the protective pathways activated by the different conditioning maneuvers in the two sexes represents a crucial point for clinical interventions.

AUTHOR CONTRIBUTIONS

GQ, MG, SA, and CP conceived the study and its design, and revised the manuscript for important intellectual content. GQ, MG, and CP wrote the manuscript. FG created the figure and revised the manuscript. All authors read, edited, and approved the final manuscript.

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MINI REVIEW
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Sex Differences of the Diabetic Heart

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Type 2 diabetes is a chronic disease associated with micro- and macro-vascular complications, including myocardial ischemia, and also with a specific and intrinsic cardiac dysfunction called diabetic cardiomyopathy (DCM). Both clinical and animal studies demonstrate significant sex differences in prevalence, pathophysiology, and outcomes of cardiovascular diseases (CVDs), including those associated with diabetes. The increased risk of CVDs with diabetes is higher in women compared to men with 50% higher risk of coronary artery diseases and increased mortality when exposed to acute myocardial infarction. Clinical studies also reveal a sexual dimorphism in the incidence and outcomes of DCM. Based on these clinical findings, growing experimental research was initiated to understand the impact of sex on CVDs associated with diabetes and to identify the molecular mechanisms involved. Endothelial dysfunction, atherosclerosis, coagulation, and fibrosis are mechanisms found to be sex-differentially modulated in the diabetic cardiovascular system. Recently, impairment of energy metabolism also emerged as a determinant of multiple CVDs associated with diabetes. Therefore, future studies should thoroughly analyze the sex-specific metabolic determinants to propose new therapeutic targets. With current medicine tending toward more personalized care of patients, we finally propose to discuss the importance of sex as determinant in the treatment of diabetes-associated cardiac diseases to promote a more systemic inclusion of both males and females in clinical and preclinical studies.

Keywords: type 2 diabetes, cardiovascular diseases, sex differences, gender differences, ischemic heart diseases, personalized care, cardioprotection, diabetic cardiomyopathy

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INTRODUCTION

An alarming report from the International Federation of Diabetes recently highlighted that prevalence of diabetes keeps increasing worldwide, affecting 463 million people in 2019 (International Diabetes Federation, 2019). Cardiovascular (CV) complications remain the predominant causes of morbidity and mortality among diabetic patients with an increased risk of heart failure, coronary artery diseases (CADs), myocardial infarction (MI), diabetic cardiomyopathy (DCM), and stroke (American Diabetes Association, 2015). Despite an estimated prevalence of diabetes slightly lower in women in comparison with men (9 vs. 9.6%) (International Diabetes Federation, 2019), strong evidence suggests worse CV consequences and mortality in diabetic women, independent of age. Consequently, there is a growing

interest for a better understanding of the molecular mechanisms involved in this phenomenon (Kautzky-Willer et al., 2016).

IMPACT OF ESTROGENS ON CARDIOVASCULAR SYSTEM

Multiple studies show that female hormones, particularly estrogens, have a beneficial effect on CV health (Dantas et al., 2012; Kander et al., 2017). Estrogen receptors ERα and ERβ are expressed in endothelial cells, vascular smooth muscle cells, and cardiomyocytes of both sexes (Cid et al., 2002; Iorga et al., 2017). Estrogens can affect lipid metabolism, energy balance, fat distribution, insulin sensitivity, and blood pressure and increase bioavailability of nitric oxide (NO) (Cid et al., 2002; Ventura-Clapier et al., 2017a). Estrogens also positively regulate vascular relaxation factors, such as prostaglandin I2. Therefore, estrogen has the ability to positively regulate CV risk factors, such as obesity, hypertension, and glucose mishandling. Studies on ovariectomized animals demonstrate greater impairment of left ventricular function following an ischemia-reperfusion episode with an implication of apoptosis, pro-inflammatory cytokines, and reactive oxygen species (ROS). Treatment with estrogens resulted in restoration of cardiac function, indicating a potential cardioprotective role of female sex hormones (Lagranha et al., 2010; Yang et al., 2018).

Compared with men, women have a higher percentage of fat mass, primarily accumulating in the subcutaneous area (Power and Schulkin, 2008). Estrogens modulate fat distribution through the expression of their receptors. Of interest, a higher ERα/ERβ ratio has been shown to correlate with lower adiposity, especially at the visceral level (Davis et al., 2013). Importantly, healthy women present lower intracardiac lipid levels than men (Huang et al., 2017), and male sex is a predictor of myocardial steatosis (Kannel and McGee, 1979; Iozzo et al., 2009). Thus, favorable distribution of fat participates in CV health in women. Another important point is the lower blood pressure from adolescence onward, due to 27% less renin system activity (Blenck et al., 2016). Hypertension is a well-known CV risk factor affecting both sexes but with higher incidence and severity in men (Kjeldsen, 2018). Endogenous estrogen maintains vasodilation, contributing to the control of blood pressure in premenopausal women (Garcia et al., 2016).

INCREASED RISK IN CARDIOVASCULAR COMPLICATIONS IN TYPE 2 DIABETIC WOMEN

Several parameters linked to sexual dimorphism could contribute to higher CV risk in type 2 diabetic (T2D) women in comparison to T2D men (**Table 1**).

A role of estrogen and its receptors has been evocated to explain the higher CV risk found in T2D women. Increased expression of ERβ compared with ERα is associated with increased oxidative stress, inflammation, and atheromatous plaque formation (Xing et al., 2009), leading to the development of type 2 diabetes and CV complications. Diabetic women present higher insulin resistance (Flanagan et al., 2006; Reichelt et al., 2013) and are more likely to be glucose intolerant, and diabetic men have elevated fasting blood glucose levels (Rydén et al., 2007). Estrogen supplementation in postmenopausal women decreases fasting blood glucose and, thus, improves glucose tolerance (Huang et al., 2017). Ovariectomized Sprague–Dawley

TABLE 1 | Sexual dimorphism in cardiovascular risk factors in absence or presence of diabetes.

	Male	Female	References
Absence of diabetes			
Lifestyle			
Food intake and energy expenditure	↑	↓	Kautzky-Willer et al., 2016
Risk of T2D with consumption of sugary drinks	–	↑	Eshak et al., 2013
Physical activity and MI risk	↓	↑	Kriska et al., 2006
Smoking and CADs risk	↓	↑	Thomas, 2017
Smoking and diabetes risk	=	=	Willi et al., 2007
Fat distribution			
Fat percent	↓	↑	Power and Schulkin, 2008
Preferential localization	Visceral	Subcutaneous	Power and Schulkin, 2008; Blüher, 2013
Ectopic cardiac fat	↑	↓	Kannel and McGee, 1979; Iozzo et al., 2009
Blood pressure			
Basal systolic and diastolic blood pressure	↑	↓	Blenck et al., 2016; Kjeldsen, 2018
Incidence and severity of HT	↑	↓	Anand et al., 2008
Cardiac adaptation to HT	Eccentric hypertrophy	Concentric hypertrophy	Krumholz et al., 1993; Santos and Shah, 2014
HF failure risk	↓	↑	Beale et al., 2018
Glucose metabolism			
Basal insulin level	↓	↑	Flanagan et al., 2006; Reichelt et al., 2013
Risk of diabetes	↑	↓	International Diabetes Federation, 2019
Presence of diabetes			
Fat distribution			
Preferential localization	Visceral	Visceral	Power and Schulkin, 2008
Risk of CADs with obesity	↓	↑	Elffers et al., 2017; Lind et al., 2017
Cardiac lipid level	↓	↑	Iozzo et al., 2009
Blood pressure			
Incidence and severity of HT	↓	↑	Anand et al., 2008
Glucose metabolism			
Manifestation	Impaired fasting blood glucose	Impaired glucose tolerance	Rydén et al., 2007
Insulin resistance	↓	↑	Flanagan et al., 2006; Reichelt et al., 2013
CV risk with prediabetes	–	↑	Levitzky et al., 2008

Arrows in the "male" column indicate differences in comparison to female; and arrows in the "female" column indicate differences in comparison to male. CV, cardiovascular; CADs, Coronary artery diseases; HF, heart failure; HT, hypertension.

females had poorer glucose tolerance than non-ovariectomized animals (Saengsirisuwan et al., 2009). Importantly, prediabetes (fasting blood glucose: 100–125 mg/dL) is predictive of CVDs only in women (Levitzky et al., 2008). The greater insulin resistance found in women, coupled with endothelial dysfunction, may explain the high risk of CV complications in T2D (Rutter et al., 2003).

Importantly, obesity increases the relative risk of CADs by 64% in women as opposed to 46% in men (Elffers et al., 2017; Lind et al., 2017). Besides this, visceral adipose tissue is the source of ectopic deposition of fat in the heart (Tchernof and Despres, 2013), participating in the development of DCM through lipotoxicity (Listenberger et al., 2001; Bugger and Abel, 2014). T2D women have a more pronounced increase in intracardiac lipid content than T2D men (Iozzo et al., 2009). The ERβ receptor prevalence results in an adipogenic and diabetogenic profile (Blüher, 2013; Davis et al., 2013), probably explaining this difference.

Rutter et al. (2003) show that the increase in left ventricular mass and wall thickness correlating to glucose intolerance is more important in women than in men, largely accounted for by obesity and pressure overload. Hypertension is more pronounced in T2D women than in T2D men, and sex appears to influence morphological cardiac adaptation to hypertension (Santos and Shah, 2014). Women tend to develop concentric hypertrophy compared with men who tend to develop eccentric hypertrophy (Krumholz et al., 1993). This is confirmed in animal models (Olsson et al., 2001). A decrease in peroxisome proliferator-activated receptor-α (PPARα) signaling is found in hypertrophic males but not in females (Harrington et al., 2017), and acute inhibition of PPARα blocks the sex difference in hypertrophy development. Accordingly, in humans suffering from aortic stenosis, Kararigas et al. (2014) reveal that cardiac hypertrophy is related to increased activation of profibrotic and inflammatory markers in men compared with women.

SEXUAL DIMORPHISM IN ISCHEMIC HEART DISEASES ASSOCIATED WITH DIABETES

In the general population, incidence of MI remains higher in men than in women. CVDs appear on average 10 years earlier in men than in women (Kannel and McGee, 1979; Thom et al., 2006; Anand et al., 2008; Dantas et al., 2012). Interestingly, women seem to lose this sex-related protection in the presence of T2D (Murphy, 2011). This could be primarily due to differences in diagnosis and treatment of MI itself. Symptoms experienced by women are, in 50% of cases, different from the classic symptoms recognized in men, such as feelings of exhaustion, digestive disorders, and shortness of breath (Mehta et al., 2016), resulting in delayed treatment (Bugiardini et al., 2017). When considering CADs, women have a 50% higher risk than men, presenting increased mortality when exposed to acute MI (Kannel, 1987; Toedebusch et al., 2018) with a strong impact of long-standing diabetes in women (Natarajan et al., 2005). Several studies show a higher risk of CADs at lower glucose levels in women (Koro et al., 2008; Levitzky et al., 2008). The Framingham study also demonstrated that risk of MI is increased by five in T2D women compared with non-diabetic women, and this risk is only multiplied by two in T2D men (Kannel et al., 1974; Wannamethee et al., 2012). Moreover, 38% of women die within 1 year of their first MI although only 25% of men do so (Thom et al., 2006).

Concomitant development of atherosclerosis, endothelial dysfunction, and impairment of the coagulation profile could explain, in part, why diabetic women present a higher risk of ischemic heart diseases (IHDs). Clinical studies reveal a more severe atherogenic dyslipidemia in diabetic women, particularly through an increase in triglycerides and lipoprotein cholesterol concentrations (Walden et al., 1984). Accumulation of oxidized low-density lipoprotein within arteries is a mechanism contributing to the development of atherosclerotic plaques. In particular, Chen et al. (2002) show that its receptor, the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), has an important role in atherosclerosis development. Interestingly, sex differences in LOX-1 are reported with a particularly high expression in diabetic and obese women (Brinkley et al., 2008), making it an interesting pathway related to sex differences in diabetes and IHDs.

Atherosclerosis is an important factor contributing to the development of endothelial dysfunction. Diabetic women are also characterized by greater endothelium impairment in comparison to diabetic men. Clinical studies particularly show endothelium-dependent vasodilation alteration, which is confirmed in different animal models of T2D (Alameddine et al., 2015; Ranucci et al., 2019). A decrease in endothelium-dependent and -independent vascular response is observed in female Goto-Kakizaki rats with lower coronary flow and reduced upregulation of the NO pathway (Desrois et al., 2017; Palee et al., 2017). Zhang et al. reveal a predisposition of females to vascular lesions after induction of diabetes in both mesenteric arteries and the aorta (Zhang et al., 2012; Hunter et al., 2017). Regulation of the protein kinase B pathway may also contribute to vascular endothelial dysfunction and myocardial sensitivity to an ischemia-reperfusion episode (Desrois et al., 2004), especially in females (Reichelt et al., 2013). Goel et al. (2008) suggest that estrogen causes gender-specific endothelial dysfunction in hyperglycemic conditions by increasing the expression of PKCβ and increasing O_2^- production in females. Hyperglycemia also alters the balance of estrogen receptors and increases both oxidative stress and the level of vasoconstrictors (Donahue et al., 2007; Wannamethee et al., 2012; Hunter et al., 2017).

Interaction between endothelial impairment and platelet aggregation is also implicated in atherosclerosis pathogenesis. Diabetic women present elevation of fibrinolytic/thrombotic factors during the transition from normoglycemia to diabetes (Steinberg et al., 2000; Donahue et al., 2007, 2011), leading to a prothrombotic coagulation profile (Steinberg et al., 2000; Donahue et al., 2007). Meigs et al. report an increase in circulating levels of thrombosis-promoting factors (Plasminogen activator inhibitor-1, von Willebrand factor) and adhesion molecules (vascular cell adhesion molecule 1, intercellular adhesion molecule 1) associated with atherosclerosis and microvascular diseases (Meigs et al., 2006; Madhu, 2010). In addition, women

with T2D are more sensitive to changes in coagulation and inflammation than men, which could be explained by the fact that women have a larger platelet count as well as higher platelet reactivity than men (Ranucci et al., 2019). Together, concomitant development of atherosclerosis, endothelial dysfunction, and impairment of the coagulation profile lead to a favorable environment for IHD development in diabetic women (**Figure 1**).

Mechanisms involved in increased mortality following myocardial infarction in diabetic women are not fully understood. Nevertheless, energy metabolism has recently emerged to explain this sex difference (**Figure 1**). A strong decrease in ATP and phosphocreatine cardiac content has been observed following ischemia-reperfusion injury in prediabetic female rats fed with a high-fat, high-sucrose diet and in diabetic GK female rats (Fourny et al., 2019a,b). Importantly a previous study reports no difference in high-energy compounds following ischemia-reperfusion injury in male GK (Desrois et al., 2010), suggesting the important role of mitochondria and particularly the energy production pathway in female sensitivity to IHDs.

SEXUAL DIMORPHISM IN HEART FAILURE ASSOCIATED WITH DIABETES

Heart failure is driven by CADs but also by aging, hypertension, diabetes, and obesity (Ho et al., 2016; Beale et al., 2018; Seferović et al., 2018). The excess risk of HF associated with diabetes is significantly greater in women with diabetes than in diabetic men (Ohkuma et al., 2019), increasing HF risk fivefold in women compared to 2.4-fold in men (Beale et al., 2018). Kim et al. (2019) also show that the impact of diabetes on long-term mortality and HF readmission seems to be greater in females than in males. Women represent ∼60% of patients having HF with preserved ejection fraction (HFpEF) whether they present with diabetes or not, but T2D women are younger, more obese, have worse renal function, lower prevalence of atrial fibrillation, and decreased hemoglobin levels (Lejeune et al., 2021). Importantly, HFpEF is more prevalent in women than in men, who preferentially exert HF with reduced ejection fraction (Beale et al., 2019; Dewan et al., 2019). In line, Weinberg et al. (1999) show sex-related differences in genes regulating calcium handling and contractile function. Males have higher beta-myosin heavy chain and atrial natriuretic factor, and lower SERCA-2 mRNAs in comparison with females despite a similar left ventricular hypertrophy and systolic overload (Weinberg et al., 1999).

Besides CADs and hypertension, the diabetic heart is characterized by alterations of its structure and mass as well as of its diastolic and systolic function, leading to the concept of DCM (Boudina and Abel, 2010). Interestingly, DCM prevalence is higher in women in comparison to men. In particular, Kiencke et al. (2010) show that female gender is an independent risk factor for DCM, characterized by greater structural and functional impairment (Kiencke et al., 2010; Toedebusch et al., 2018; **Figure 1**).

Myocardial remodeling occurs during DCM development with an increase in fibrosis and collagen I and III deposits, leading to myocardial rigidity (Murphy et al., 2017). Studies show greater myocardial remodeling and fibrosis in women with HF compared with men (Li et al., 2017). Women with T2D have greater cardiac hypertrophy, myocardial wall thickening, and an increase in left ventricular mass. Greater hypertrophy is also observed in female GK rats compared with males (Desrois et al., 2004). Estrogen receptor ERβ is shown to play an important role in the regulation of collagen levels (Schuster et al., 2016). Schuster et al. (2016) reveal that overexpression of ERβ in mice reduced myocardial fibrosis and collagen I/III deposits, improving cardiac function. Inversely, Skavdahl et al. (2005) detect basal cardiac hypertrophy in female mice deficient for ERβ, confirming the important role of this receptor for cardiac hypertrophy development in females. Moreover, imbalance between ERα and ERβ is reported in diabetic women and may explain the loss of estrogen cardioprotection regarding myocardial hypertrophy and fibrosis in DCM (Wells et al., 2005).

A fined-tuned regulation of metabolism and energy production is essential for heart function (Horman et al., 2012; Bertrand et al., 2020). Female cardiomyocytes contain

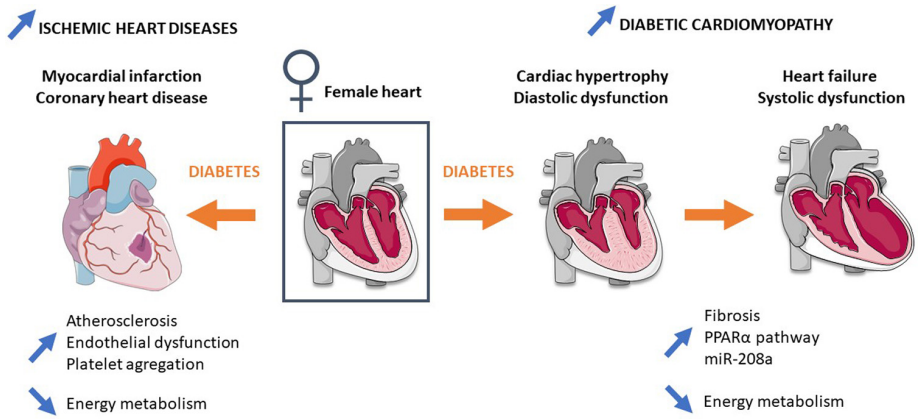

FIGURE 1 | Mechanisms involved in the higher risk and mortality of type 2 diabetic women in ischemic heart diseases and diabetic cardiomyopathy. Detailed information is provided in the main text. Blue arrows represent differences in comparison to diabetic men.

less mitochondria, but they are more efficient than in male cardiomyocytes. Female hearts use fatty acids for energy production in greater proportion than males (Djouadi et al., 1998; Ventura-Clapier et al., 2017b). They also produce fewer ROS, have a lower calcium uptake rate, and a greater calcium retention capacity (Ventura-Clapier et al., 2017b). This sexual dimorphism does not lead to a difference in respiration and mitochondrial efficiency in the basal state but could play a role in pathological situations such as DCM. Of interest, a mitochondrial localization of estrogen receptors is reported, inducing direct effects of estrogen on mitochondrial respiration and antioxidant defenses (Gupte et al., 2015). Billimoria et al. (2006) show a greater mitochondrial respiration in female streptozotocin-treated (STZ) rats in comparison with corresponding males. Lagranha et al. (2010) show that the phosphorylation level of mitochondrial proteins is more important in females compared with males. This is particularly the case for the aldehyde dehydrogenase 2, leading to a decrease in ROS production (Lagranha et al., 2010; Tchernof and Despres, 2013).

Metabolic inflexibility is commonly noticed in the diabetic heart, which mainly relies on fatty acid oxidation for energy production (Vallerie and Bornfeldt, 2015). The increase in fatty acid oxidation in the diabetic heart is associated with an increase in PPARα, which plays a key role in the development of cardiac hypertrophy and dysfunction in DCM (Madrazo and Kelly, 2008; Bayeva et al., 2013). Interestingly, estrogen is involved in the signaling pathway of lipid metabolism and may explain the differences in mitochondrial metabolism observed between diabetic males and females. Indeed, Djouadi et al. (1998) show, in PPARα$^{-/-}$ mice, that the subsequent inhibition of cellular fatty acid metabolism caused massive accumulation of hepatic and cardiac lipids, hypoglycemia, and death in 100% of males but only 25% of females. The treatment with β-estradiol decreased the mortality in males, demonstrating the role of female sex hormones in lipid homeostasis mediated by PPARα (Djouadi et al., 1998). In the last decade, micro-RNAs emerged as biomarkers of DCM and targets for new treatment. Yin et al. (2019) show that miR-30c protects cardiac metabolism and function in diabetes through PPARα modulation and its downstream effector, the co-activator Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α). The miR-208a, whose overexpression induced spontaneous cardiac hypertrophy (Callis et al., 2009), is another miR playing a role in DCM. Recently, Lum-Naihe et al. (2017) highlighted higher miR-208a expression in female diabetic hearts than in male counterparts.

PERSONALIZED CARE OF DIABETIC PATIENTS

Involvement of female hormones in various physiological and pathophysiological processes has led the scientific community to focus their research on the male sex. However, we currently know that women have a different clinical presentation and drug response in multiple pathologies, including CVDs and T2D (Mathieu et al., 2018; Fourny et al., 2019b). First, clinical trials demonstrated sex differences in lifestyle intervention in diabetic patients. Moderate-intensity resistance exercise training is a more favorable approach for hypertensive women because of greater decreases in diastolic blood pressure and significant increases in flow-mediated dilation compared with their male counterparts (Collier et al., 2011). Weight loss with intensive lifestyle modification led to greater decreases in glucose/insulin concentration, insulin resistance, triglyceride, and glycated hemoglobin HbA1c levels in men than in women, indicating that women should particularly pay attention to risk factors, such as obesity (Perreault et al., 2008). This was confirmed in animal models in which diet change is most effective to reduce inflammation in male mice (Griffin et al., 2019).

Sex differences are also reported in regard to the response to antidiabetic drugs. In young patients, metformin plus rosiglitazone was more effective in girls than in boys (Zeitler et al., 2012). In adults, women had a higher reduction of body weight after treatment with metformin or sulfonylurea, whereas men displayed higher HbA1c reduction after treatment with metformin only (Schütt et al., 2015). Sex differences were also reported for incretins with a better glycemic control in men (Anichini et al., 2013) while others showed greater weight loss, reductions of fasting glucose, and blood pressure levels in women (Pencek et al., 2012). The LEADER study highlighted a greater CV benefit in men than in women with liraglutide treatment (Verma et al., 2018). Recently, Raparelli et al. (2020) demonstrated greater CV effectiveness of GLP-1 receptor agonist in women. However, "the real-world experience" study showed that men achieved target glycemic response in higher proportions than women after 1 year of exenatide (Anichini et al., 2013). A greater glycemic response and HbA1c reduction was found with sulfonylureas than with thiazolidinediones in men, whereas female sex was associated with greater HbA1c reduction but a weight gain and edema risk with thiazolidinediones (Dennis et al., 2018). Interestingly, Zinman et al. (2015) reported no sex difference in the EMPA-REG OUTCOME trial in effects of a sodium-glucose cotransporter 2 inhibitor. However, subgroup analysis showed a significant CV benefit in males only (Kautzky-Willer and Harreiter, 2017). In 2020, an important study showed no differences for vascular efficacy outcomes or death with major protection against major adverse CV events, HF, vascular death, and total mortality in both men and women (Rådholm et al., 2020). Taken together, these studies clearly show that sex should be considered in the choice of antidiabetic treatment to move toward "precision medicine," which aims to treat patients with accurate care that is more personalized and including individual variability (Currie and Delles, 2018; Prasad and Groop, 2019). However, mechanisms involved in these differences are not yet understood, and disparity of antidiabetic treatments used, alone or in combination, makes comparison difficult.

The choice of the animal model to be employed is also delicate for the efficient transfer of results to humans, particularly when comparing males and females. Indeed, studying females is not always possible in animal T2D models. For example, the female TallyHo mouse does not show hyperglycemia unlike males (Kim et al., 2005) and the female Nagoya-Shibata-Yasuda mouse has a low incidence of type 2 diabetes compared with

males (Ueda et al., 1995). Besides this, enriched diets are also commonly used in the literature, but their diversity and duration make comparison difficult. Thus, each diet-induced and energetic diabetic model should be well characterized to ensure good interpretation of the results obtained in both sexes.

CONCLUSION

In conclusion, clinical and animal studies clearly indicate that there are sex differences in T2D-associated CV complications. However, the precise molecular mechanisms responsible for these differences are still largely blurred. Recent studies have particularly emphasized the link between energy metabolism and miRs. Thus, future studies should particularly pay attention to the metabolic dysfunctions that are involved in both IHDs and DCM development. This could provide new targets for the treatment of the diabetic heart. In addition, the antidiabetic drug response also differs significantly according to sex. Therefore, the scientific community must include both sexes in future clinical trials and animal studies to improve quality of care and bring a more personalized treatment to each patient.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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BRIEF RESEARCH REPORT
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Heart Angiotensin-Converting Enzyme and Angiotensin-Converting Enzyme 2 Gene Expression Associated With Male Sex and Salt-Sensitive Hypertension in the Dahl Rat

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Angiotensin-converting enzyme 2 (ACE 2) in the heart including its sex dependency in the hypertensive heart, has not been much studied compared to ACE. In the present study, we used the Dahl salt-sensitive rat exposed to fructose and salt to model a hypertensive phenotype in males, females, and ovariectomized females. Blood pressure was measured by the tale-cuff technique in the conscious state. Expression of RAS-related genes ACE, ACE2, angiotensin II receptor type 1, Mas1, and CMA1 in the heart were quantified. The results revealed small but significant differences between male and female groups. The main results indicate the presence of a male preponderance for an increase in ACE and ACE2 gene expression. The results are in accordance with the role of androgens or male chromosomal complement in controlling the expression of the two ACE genes.

Keywords: hypertensive heart, angiotensin-converting enzyme, male, female, rat, salt-sensitive, fructose, angiotensin-converting enzyme 2

INTRODUCTION

The COVID-19 pandemic has drawn attention to the connection between cardiovascular disease and coronavirus infections. Hypertension seems to increase the risk of symptomatic COVID-19 infection. Male sex is overrepresented among those with severe disease development and fatality (Borges do Nascimento et al., 2020; Gebhard et al., 2020). Male and female sex hormones are steroids with the regulation of gene expression as their main (but not only) mechanism of action. Sex steroid-responsive elements are present in various cells of the heart.

Angiotensin-converting enzyme 2 (ACE2) (Gheblawi et al., 2020), the cell membrane ectopeptidase linked to the renin-angiotensin system (RAS), is described to function as a receptor for the penetration of the coronavirus into human cells (Chen et al., 2020).

Major RAS components are widely expressed in the body and are important treatment targets against hypertension. Angiotensin II (AngII) produced from angiotensin I (AngI) by the enzyme ACE or chymase, has vasoconstrictive, proinflammatory, and prooxidative effects *via* binding to the AT1R receptor (ACE1/AngII/AT1R axis). An alternative pathway is the conversion of AngI or AngII to the peptide Ang1-7 by the ACE2 enzyme. Ang1-7 binds to a specific MAS receptor promoting anti-fibrotic and vasodilator effects counter-regulating the effect of AngII (ACE2/Ang1-7/Mas axis). Ang1-9 is an alternative product of AngI with an anti-hypertropic effect. At the protein level, ACE is constitutively present in endothelial cells independent of the organ. Proposed cellular sources of ACE2 in the heart are macrophages and in the vessel wall most likely pericytes and cardiomyocytes (Burrell et al., 2005; Chen et al., 2020; Hikmet et al., 2020). The Mas receptor is reported to be present in the sarcolemma of cardiomyocytes (Bader et al., 2018). Chymase in mast cells localized to the heart represents renin and ACE independent pathway to AngII production (Ferrario et al., 2020; Froogh et al., 2020).

The present study aimed to examine if upstream regulation of ACE2 and related RAS gene-expression components in the hypertensive heart were dependent on sex in a female sex hormone-reliant manner. For this purpose, we examined male, female, and ovariectomized female Dahl salt-sensitive hypertensive rats. Only minor differences in gene expression of RAS components were detected. However, male sex but not female sex or loss of ovary function was associated with higher ACH2 and ACE gene expression.

METHOD

Six groups ($n = 14$–15) of adult male and female Dahl salt-sensitive rats, aged 12 weeks, were included in the study. The female rats were divided into two groups, one group underwent ovariectomy, and thereafter, the Dahl rats received fructose in their drinking water with a control diet (0.3% NaCl) or an elevated salt diet (6%). The study was approved by local and Norwegian animal welfare authorities (approval ID 6784), and all procedures conformed to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Blood pressure was monitored non-invasively using the tale-cuff technique. After 8 weeks, heart samples from the apex of the left ventricle were harvested and stored in RNA later (Qiagen, Hilden, Germany) and expression of selected target genes ACE, ACE2, Agtr1, MAS1, and CMA1 were examined (**Table 1**). Total RNA was isolated according to the RNeasyFibrous Tissue protocol (Qiagen). RNA concentration was measured spectrophotometrically (NanoDrop, Witec, Switzerland). Reverse transcription of RNA was carried out using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The qRT-PCR was performed in an ABI PRISM 7900 HT Fast real-time thermal cycler using the SYBR green master mix (Applied Biosystems). Primers were obtained from Eurogentec (Seraing, Belgium) and Sigma-Aldrich (St Louis, Mo, USA). The relative expression ratio of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method. The expression of the target genes was normalized to stably expressed reference genes succinate dehydrogenase complex flavoprotein subunit A (SDHA) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) selected based on testing by NormFinder (Andersen et al., 2004).

The statistics were based on a 2×3 factorial design with two independent variables, diet-salt two levels (with and without salt) and sex three levels (male, female, and ovariectomized female), respectively. Statistical analysis was performed using two-way ANOVA (SigmaStat, Systate Software) for the effect of sex and the effect of increased diet salt and interaction between these two. The Holm-Sidac test was used as a *post-hoc* test to test for significance between the six groups. Results are presented as mean ± SEM.

TABLE 1 | List of primers for gene expression analysis with their corresponding protein names.

Gene	Protein		Primer
HPRT1 (HPRT)	Hypoxanthine-guanine phosphoribosyltransferase	NM_012583.2	GACCGGTTCTGTCATGTCG
			ACCTGGTTCATCATCACTAATCAC
SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein variant	NM_130428.1	CCCTGAGCATTGCAGAATC
			CATTTGCCTTAATCGGAGGA
Ace (ACE1)	Angiotensin I converting enzyme	NM_012544.1	GGAGACGACTTACAGTGTAGCC
			CACACCCAAAGCAATTCTTC
Ace2	Angiotensin I converting enzyme 2	NM_001012006.1	TCAAGGGAAAAGAACCAGACA
			GGTTTCAAATCACTCACCCATAC
Agtr1	Rattus norvegicus angiotensin II receptor type 1, type 1b (Agtr1b)	NM_031009.2	GGTTCAAAGCCTGCAAGTGAA
			GAGTGAGCTGCTTAGCCCAAA
Cma1 (CYH; MCT1; chymase)	chymase 1, mast cell	NM_013092.1	ACTCTCGGCCAACTTCAACT
			TTCACGTTTGTTCTGCCCCA
Mas1	MAS1 proto-oncogene, G protein-coupled receptor	NM_012757.2	GGAGAGCCTGATTTCCCCTC
			ACAGTGAGCTGGGTGCTTTG

TABLE 2 | Blood pressure, body weight, and heart weight (indexed to tibia length).

	Male	Male salt	Female	Female salt	Female OVX	Female OVX salt
Body weight (g)	407 ± 5^{+}	393 ± 7^{+}	254 ± 2	260 ± 4	293 ± 8^{+}	306 ± 2^{+}
Heart/tibia (g/cm)	2.5 ± 0.09^{+}	2.6 ± 0.07^{+}	1.9 ± 0.04	2.2 ± 0.07*	2.1 ± 0.06	2.5 ± 0.06^{+*}
BP systole (mmHg)	156 ± 4.5	186 ± 6.3*	161 ± 7.7	183 ± 6.9*	153 ± 4.0	192 ± 6.2*
BP diastole (mmHg)	111 ± 4.3	139 ± 6.1*	117 ± 7.9	138 ± 6.7*	100 ± 3.6	143 ± 7.5*

*Mean ± SEM (n = 14–15), *indicates $p < 0.05$ vs. the corresponding same-sex group with normal salt, $^{+}$indicates $p < 0.05$ vs. the corresponding female ovary-intact normal diet group.*

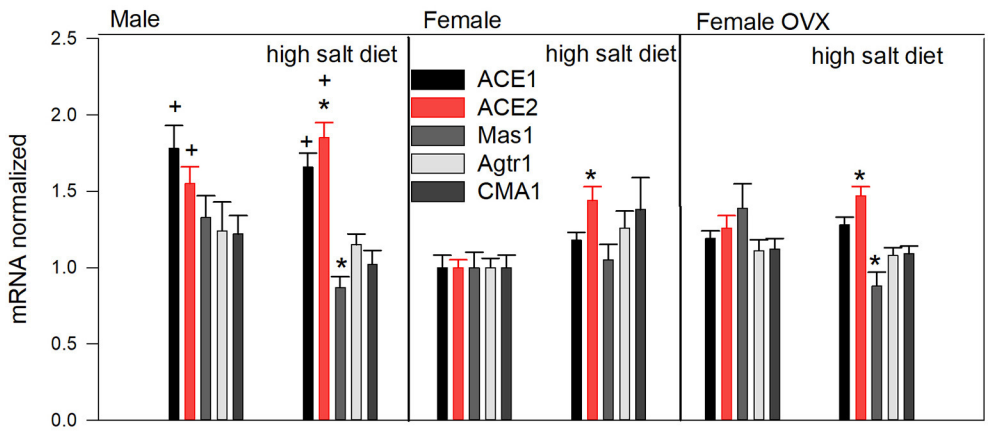

FIGURE 1 | Expression of selected genes (mRNA) normalized to housekeeping genes succinate dehydrogenase complex flavoprotein subunit A (SDHA) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) and presented relative to the expression level in female ovary-intact hearts with standard salt diet. Mean ± SEM (n = 14–15), *indicates $p < 0.05$ vs. the corresponding same-sex group with normal salt, $^{+}$indicates $p < 0.05$ vs. the corresponding female ovary-intact normal diet group (*this group serving as control*).

RESULTS

At the endpoint, blood pressure in the six groups was elevated above values at 12 weeks in untreated low salt diet Dahl rats (historical controls, mean arterial pressure males 101 ± 3.4 mmHg, females 105 ± 3.2, ovx females 102 ± 4.0, $n = 6$). The added salt diet significantly increased both systolic and diastolic arterial blood pressure above the fructose alone (**Table 2**). Interestingly, there were no differences in blood pressure between the three subgroups within each of the two diet groups i.e., there were no detectable differences in blood pressure between male, female, and female ovariectomized rats suggesting that any difference in gene expression would not be a direct reflection of differences in blood pressure at the endpoint. However, a significant difference in heart weight relative to tibia length was present between females given elevated salt and females given standard salt.

Angiotensin-converting enzyme 2 gene expression increased slightly but significantly in all three salt-exposed hypertensive groups (**Figure 1**). Interestingly, the highest level of ACE2 expression was observed in high salt male hypertensive hearts, which was almost doubled compared to hearts from females with a standard salt diet (1.85 ± 0.1 vs. 1.00 ± 0.05 units). With respect to ACE1, expression was more pronounced in males compared to female hearts, independent of diet intervention. Mas1 tended to be slightly downregulated in hypertensive hearts, however, only significantly in hearts from males and ovariectomized females when compared to corresponding controls.

The gene expression data are presented relative to the expression of the same gene in ovary intact females exposed to normal salt (**Figure 1**). Normalized to HPRT and STDA measured values were ACE 0.034 ± 0.002, ACE2 0.0032 ± 0.00017, Agtr1α 0.025 ± 0.0016, Mas1 0.0023 ± 0.0024, and CMA1 0.0032 ± 0.0037 (mean ± SEM) in this subgroup. The calculated average ratio ACE2/ACE gene expression was 0.09 ± 0.008 in males, 0.106 ± 0.011 in females, and 0.102 ± 0.007 in female ovx and in corresponding subgroups receiving high salt 0.110 ± 0.008, 0.120 ± 0.009, and 0.112 ± 0.005 ($p = 0.036$, overall effect of the high salt intervention).

DISCUSSION

Salt-dependent hypertension is widely distributed in the human population and is caused by the interaction between diet and individual genetic makeup (Rodriguez-Iturbe et al., 2007). The condition is mimicked experimentally in the Dahl salt-sensitive rat, which gradually develops hypertension on a standard diet supplemented with fructose and accelerates its development on the high salt diet (Ludvigsen et al., 2018; Lee et al., 2020). In the present study of RAS-related gene expression in Dahl salt-sensitive male and female rat hearts, we observed that

overall expression of ACE and ACE2 was higher in males with elevated blood pressure compared to females. Two levels of elevated blood pressure were studied, and the more pronounced hypertension increased ACE2 gene expression slightly but significantly in all three groups. Interestingly, we did not find significant differences between hearts from intact females and ovariectomized females. Thus, the presence of androgens and/or other compounds related to the difference in sex chromosome patterns between males and females might be responsible for slightly higher ACE and ACE2 expression in males in the present study.

Angiotensin-converting enzyme 2 is an X-chromosome linked in both humans and rats. In the case of functionally significant ACE2 polymorphisms in the population, males depend on their maternal variant of the gene, whereas females are mosaic and can be more robust or phenotypically variable. In humans, it is proposed that the ACE2 belongs to a part of the X chromosome that escapes inactivation. To our knowledge, it is not known if this is the case in the rat. In accordance with a connection between androgens and ACE2 expression, ACE2 is present in the male reproductive tract and is especially highly expressed in the testis (Younis et al., 2020). Conclusions from the present study are substantially limited since differences in expression were statistically significant but small, and their physiological relevance appears therefore unclear. Gene expression changes do not predict ACE2 level at the cell surface. We cannot deduce that male hearts have more receptors for the coronavirus; however, we conclude that the regulation at gene expression level by male sex or androgens needs to be examined in different experimental models. Interestingly, the results based on gene expression in the Dahl salt-sensitive rat is in agreement with findings from a study undertaken in spontaneously hypertensive rats measuring ACE and ACE2 activity in male and female hypertensive hearts (Dalpiaz et al., 2015).

Compared to ACE, the ACE2 gene is expressed at a low level, and the overall significance of ACE in the male heart should not be underscored. It has repeatedly been shown that ACE inhibition and AngII receptor antagonism are effective treatments against blood pressure elevation and the progression of concomitant observed heart hypertrophy and remodeling in the Dahl rat (Kim et al., 2001). Heart hypertrophy, fibrosis, and inflammation are the main findings when salt is started at an early age (7 weeks) leading to cardiac failure mirroring heart failure with preserved ejection fraction (HFpEF) after 10 weeks (Gallet et al., 2016). With respect to experimental studies of pressure-induced heart remodeling, most studies are performed in the male. However, in heart failure mast cells, macrophages and T cells contribute significantly and add to the complexity regarding the role of chromosomal sex and sex hormones. This study does not give any evidence to propose a cell source of the various components of RAS in the heart. CMA1 was included since mast cell is part of a hypertensive remodeling of the heart, chymase is a pathway for ACE independent conversion of AngI to AngII in the heart, and sexual dimorphism has been proposed with estrogen as a regulating factor (Li et al., 2015). The decrease in detected expression in high salt male hearts and female ovariectomized hearts is minor, and all hearts studied were obtained from fructose-feed rats with elevated blood pressure (Tran et al., 2009).

In conclusion, the increased expression level of ACE and ACE2 found in the male hypertensive heart indicates that more attention should be paid to mechanisms regulating the different parts of RAS by taking both hormonal status and sex into consideration.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by The Norwegian animal welfare authorities. FDU ID 6784.

AUTHOR CONTRIBUTIONS

KY responsible for conception and design of the study, responsible for interpretation of the results, and drafted the manuscript. SL designed and performed the experiments, analyzed the data and drafted figures, and participated in interpretation. CM participated in developing the experimental model, analyzed data, and contributed to preparation of the manuscript as well as interpretation of results. GS and EG were responsible for conception and design of the study as well as interpretation of results. All authors contributed to the article and approved the submitted version.

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Potentiation of Acetylcholine-Induced Relaxation of Aorta in Male UC Davis Type 2 Diabetes Mellitus (UCD-T2DM) Rats: Sex-Specific Responses

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Previous reports suggest that diabetes may differentially affect the vascular beds of females and males. The objectives of this study were to examine whether there were (1) sex differences in aortic function and (2) alterations in the relative contribution of endothelium-derived relaxing factors in modulating aortic reactivity in UC Davis Type 2 Diabetes Mellitus (UCD-T2DM) rats. Endothelium-dependent vasorelaxation (EDV) in response to acetylcholine (ACh) was measured in aortic rings before and after exposure to pharmacological inhibitors. Relaxation responses to sodium nitroprusside were assessed in endothelium-denuded rings. Moreover, contractile responses to phenylephrine (PE) were measured before and after incubation of aortic rings with a nitric oxide synthase (NOS) inhibitor in the presence of indomethacin. Metabolic parameters and expression of molecules associated with vascular and insulin signaling as well as reactive oxygen species generation were determined. Diabetes slightly but significantly impaired EDV in response to ACh in aortas from females but potentiated the relaxation response in males. The potentiation of EDV in diabetic male aortas was accompanied by a traces of nitric oxide (NO)- and prostanoid-independent relaxation and elevated aortic expression of small- and intermediate conductance Ca^{2+}-activated K^{+} channels in this group. The smooth muscle sensitivity to NO was not altered, whereas the responsiveness to PE was significantly enhanced in aortas of diabetic groups in both sexes. Endothelium-derived NO during smooth muscle contraction, as assessed by the potentiation of the response to PE after NOS inhibition, was reduced in aortas of diabetic rats regardless of sex. Accordingly, decreases in pAkt and peNOS were

observed in aortas from diabetic rats in both sexes compared with controls. Our data suggest that a decrease in insulin sensitivity *via* pAkt-peNOS-dependent signaling and an increase in oxidative stress may contribute to the elevated contractile responses observed in diabetic aortas in both sexes. This study demonstrates that aortic function in UCD-T2DM rats is altered in both sexes. Here, we provide the first evidence of sexual dimorphism in aortic relaxation in UCD-T2DM rats.

Keywords: sex differences, aorta, type-2 diabetes, nitric oxide, insulin resistance

INTRODUCTION

Over the past decade, obesity and type 2 diabetes (T2D) have reached epidemic levels worldwide, becoming one of the most challenging health problems in the 21st century (Tabish, 2007; Zheng et al., 2018). Cardiovascular diseases (CVDs) are one of the primary causes of morbidity and mortality in patients with diabetes (Brunner et al., 2005). Premenopausal women have a lower incidence of CVD when compared with age-matched men (Barrett-Connor, 1994). However, premenopausal women with diabetes not only lose the sex-based cardiovascular protection but also experience a higher relative risk of CVD compared to diabetic men (Steinberg et al., 2000). It has been established that hyperglycemia and diabetes affect female and male vascular beds differently. We previously reported sex differences in the development of vascular dysfunction in arteries of streptozotocin-induced type 1 diabetic rats (Zhang et al., 2012; Han et al., 2016). However, the pathophysiology of vascular dysfunction in T2D is likely to differ from that in type 1 diabetes.

With the increasing prevalence of T2D, creating effective preclinical models of the disease has become crucial for disease prevention and treatment. Dr. Havel and colleagues at the University of California (UC) Davis developed a validated rat model of T2D, the UC Davis Type 2 Diabetes Mellitus (UCD-T2DM) rats. UCD-T2DM rats exhibit all of the key features of the disease in humans such as polygenic adult-onset obesity, insulin resistance, intact leptin signaling, and spontaneous β-cell decompensation with preserved fertility in both sexes (Cummings et al., 2008; Kleinert et al., 2018).

Recently, we reported sex differences in the development of impaired vascular reactivity in mesenteric arteries from UCD-T2DM rats (Shaligram et al., 2020). Nevertheless, it remains to be established whether this reported sexual dimorphism is specific to the small arteries or is generalizable to larger conduit arteries in type 2 diabetic arteries. Thus, the initial aim of our study was to determine whether the aortic response to endothelium-dependent and independent vasodilators and vasoconstrictors varies with sex in UCD-T2DM rats.

Endothelium-dependent vasorelaxation (EDV) is considered a reproducible factor for assessing endothelial function (De Vriese et al., 2000). In diabetes, enhanced (Aloysius et al., 2012), impaired (Vanhoutte et al., 2009), and preserved EDV (Miller and Vanhoutte, 1991) have been reported. Altered EDV can result from alteration in synthesis or release of endothelium-derived relaxing factors (EDRF) [nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF)] or endothelium-derived contracting factors.

Nitric oxide has been considered a major contributor to EDV in large conduit arteries (Félétou, 2011), whereas EDHF plays a predominant role in small resistance arteries (Garland et al., 1995). In large conduit arteries, it is widely accepted that NO levels are reduced in diabetes (Han et al., 2014) and that changes in the level of endothelial NO synthase (eNOS) and/or increased generation of reactive oxygen species (ROS) such as superoxide may contribute to the reduction of NO production or bioavailability.

It has also been proposed that EDHF may play a role as a backup vasodilator in small resistance vessels when NO bioavailability is compromised (Brandes et al., 2000). The chemical identity of EDHF varies with vascular size, vascular bed and species (Leo et al., 2011). The classical EDHF pathway involves the opening of small- and intermediate-conductance calcium-activated potassium channels (SK_{Ca} and IK_{Ca}) on the endothelium and the subsequent hyperpolarizing of smooth muscle cells *via* activation of Na-K-ATPase and/or K_{ir} channels or through myoendothelial gap junctions (Edwards et al., 1998; Parkington et al., 2002; Sandow et al., 2002). Previous studies have provided evidence of EDHF-type responses induced by acetylcholine (ACh) in rabbit conduit arteries that are potentiated by the elevation of cAMP but inhibited by disruption of gap junctions or a combination of SK_{Ca} and IK_{Ca} channel blockers (Griffith et al., 2002).

Overall, studies in various experimental models have evaluated the effects of diabetes on endothelial NO production. However, the sex-specific effects of T2D on the relative contribution of EDRF to the vascular reactivity of large conduit arteries remain unclear. Here, we examined changes in the relative importance of EDRF in modulating aortic relaxation in male and female UCD-T2DM rats.

Insulin resistance, a key element in the pathogenesis of T2D (Ormazabal et al., 2018), is associated with endothelial dysfunction by several mechanisms including oxidative stress. Here, we evaluated the responsiveness to insulin signaling by measuring the aortic expression of insulin receptor substrates (IRS-1 and IRS-2), total and phosphorylated levels of Akt, and eNOS. Since NADPH oxidases (NOX) are a potent cellular source of superoxide in the cardiovascular system (Drummond and Sobey, 2014), experiments were also carried out to determine the aortic expression of NOX subtypes. Moreover, ROS generation was determined in primary aortic endothelial cells isolated from male and female UCD-T2DM rats.

This study demonstrates that aortic function in UCD-T2DM rats is altered in both sexes. Here, we provide the first evidence of sexual dimorphism in aortic relaxation in UCD-T2DM rats.

MATERIALS AND METHODS

Experimental Animals

Male and female UCD-T2DM rats were generated by breeding obese Sprague–Dawley (SD) rats with Zucker Diabetic Fatty (ZDF) lean rats that were homozygous wild type for the leptin receptor and had inherent β-cell defects. Rats were bred at the animal facility in the Department of Nutrition at the UC Davis (Cummings et al., 2008).

Rats were maintained with water and standard rodent chow food *ad libitum* at constant humidity and temperature, with a light/dark cycle of 12 h. After acclimation for 1 week at the animal facility at the University of the Pacific, animals were euthanized for experiments using carbon dioxide as the euthanasia agent, according to the recommendations from the 2013 AVMA Guidelines on Euthanasia and the NIH Guidelines for the Care and Use of Laboratory Animals: Eighth Edition (US National Institutes of Health, 2011). Age-matched male and female non-obese and non-diabetic SD rats (Simonsen Laboratories, Gilroy, CA, United States) (∼average 19–20 weeks old) were employed as controls for UCD-T2DM rats. Diabetic phase was determined by measuring blood glucose levels for three subsequent measurements using a standard glucose test meter (OneTouch, LifeScan, CA). Animals were considered diabetic when non-fasting blood glucose levels were higher than 300 mg/dl. The diabetic animals used in the study were diabetic for 35 ± 2.7 (males) and 31 ± 3.1 (females) days. UCD-T2DM rats exhibit insulin resistance prior to the onset of diabetes, similar to humans (Cummings et al., 2008).

All animal protocols were approved by the Animal Care Committee of the University of the Pacific and UC Davis Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals: Eighth Edition (US National Institutes of Health, 2011) and with ARRIVE guidelines.

Measurement of Metabolic Parameters in the Plasma

Blood glucose levels were measured in 12-h fasted rats using a standard glucose test meter (OneTouch, LifeScan, CA) and triglycerides were measured by using an Accutrend Plus System (hand-held point-of-care device) and specific test strips (Roche Farma, Barcelona, Spain) with a drop of blood collected from the tail vein. Blood samples were collected from intracardiac puncture and obtained in tubes containing heparin as an anticoagulant. Plasma was obtained by centrifugation at 10,000 × *g* for 5 min at 4°C and stored at −80°C until used. Insulin levels were determined in plasma samples by using ELISA kits according to the manufacturer's protocol (Spi Bio, Montigny Le Bretonneux, France). Insulin sensitivity index (ISI) was determined from fasting plasma glucose and insulin using the following formula: ISI = [2/(blood insulin (nM) × blood glucose (μM) + 1)] (Sangüesa et al., 2017). HbA1c was measured using the Bayer A1cNow test kit, according to the manufacturer's instructions, and animals with A1c levels greater than 6.5% on two separate tests were considered diabetic.

Measurement of Arterial Tension

The thoracic aorta was cut into 2-mm rings after being excised and cleaned off adhering connective tissues. To measure isometric tension, the rings were suspended horizontally between two stainless steel hooks in individual organ baths containing 20 ml of Krebs buffer (in mM: 119 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 24.9 $NaHCO_3$, 0.023 EDTA, 1.6 $CaCl_2$, and 6.0 glucose) at 37°C bubbled with 95% O_2 and 5% CO_2. Isometric tension was continuously monitored with a computer-based data acquisition system (PowerLab; ADInstruments, Colorado Springs, CO, United States). To develop a stable basal tone, aortic rings were equilibrated under 1*g* resting tension for 40 min. Rings were stimulated two times with 80 mM KCl every 20 min until maximum contraction was achieved. The ability of ACh (10 μM) to induce relaxation of phenylephrine (PE, 2 μM) pre-contracted vessels was taken as evidence for the preservation of an intact endothelium. For the relaxation studies, an equal submaximal concentration of PE (2 μM) was used in both males and females.

Relaxation Responses to ACh

Aortic rings were precontracted with PE (2 μM), which represented a concentration that produced 80% of the maximal effect (EC_{80}). The concentration response curves (CRCs) were obtained by the addition of increasing concentrations of ACh (10^{-8} to 10^{-5} M). In addition, CRCs to ACh were obtained before and after 20 min incubation with indomethacin (Indo; 10 μM; dissolved in DMSO), a cyclooxygenase (COX) inhibitor, Indo plus 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ; 10 μM), an inhibitor of soluble guanylate cyclase (sGC), and finally after incubation with Indo, ODQ, and *N*-nitro-L-arginine (L-NNA; 100 μM), a non-selective NO synthase (NOS) inhibitor. Tissues were washed with Krebs buffer between each CRC to allow the rings to return to basal tone.

Relaxation Responses to Sodium Nitroprusside (SNP)

CRCs to SNP (10^{-9} to 10^{-5} M), a NO-donor, were obtained in endothelium-denuded aortic rings pre-contracted with PE (2 μM) taken from all experimental groups.

Contractile Responses to PE

The constrictor CRCs to PE (10^{-8} to 10^{-5} M) were generated before and after incubation with N^{ω} -nitro-L-arginine methyl ester (L-NAME, 200 μM), a NOS inhibitor, in the presence of Indo (10 μM, dissolved in DMSO), a COX inhibitor. Tissues were washed with Krebs buffer between each CRC to allow the rings to return to basal tone. A vehicle-only (no drugs present) study was performed simultaneously in aortic rings from the same animal (data not shown).

Western Blot Analysis

Aortic tissue samples were micronized through freezing with liquid nitrogen and grinding with a mortar and pestle as previously described (Baena et al., 2015). To obtain total protein extract, samples were incubated with RIPA buffer (Sigma-Aldrich, St. Louis, MO, United States) containing Protease Inhibitor Cocktail (UltraCruz, Santa Cruz Biotechnology, Dallas, TX, United States) for 1.5 h at 4°C and centrifuged at 15,000 × *g* for 15 min at 4°C, and supernatants were collected. Protein concentrations were determined by the bicinchoninic acid (BCA) assay.

Protein (20–30 μg) was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to 0.45-μm nitrocellulose membranes (Bio Rad Laboratories Inc., Hercules, CA, United States), blocked for 1 h at room temperature with 5% w/v BSA in 0.1% Tween-20 Tris-buffered saline (TBS), and incubated overnight at 4°C with primary antibodies. All primary antibodies were diluted 1:1000 unless otherwise noted. Primary antibodies for endothelial NO synthase (eNOS), phospho-eNOS (p-eNOS) (Ser-1177), V-akt murine thymoma viral oncogene homolog-2 (Akt), phospho-AKT(p-AKT) (Ser-473), and insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) were supplied by Cell Signaling (Boston, MA, United States). Antibodies against NOX1, NOX4, KCNN3 (SK_{Ca}), and KCNN4 (IK_{Ca}) were obtained from Abcam (Cambridge, MA, United States). Incubation with secondary antibody (LI-COR donkey anti-Rabbit IgG IRDye 680 or anti-mouse IgG IRDye 800CW, 1:10,000) was performed in the blocking buffer for 1 h at room temperature. Before analyzing, the membrane was washed four times with TBS containing 0.1% Tween-20. Detection was done by using a LI-COR Odyssey imaging system (Lincoln, NE, United States). The bands were quantified by densitometry using Image Studio Lite software. To confirm the uniformity of protein loading, blots were incubated with GAPDH and β-actin antibodies (Cell Signaling, Boston, MA, United States) and were normalized for GAPDH and β-actin (data expressed as fold change from control group).

Measurement of ROS Generation by Rat Aortic Endothelial Cells

Primary Cell Isolation and Culture

Aortas were cut open lengthwise to expose the endothelial surface. Vessels were incubated in a collagenase II (Worthington) solution (2 mg/ml in DMEM) at 37°C with the endothelial surface facing down for 30 min. Collagenase was blocked 1:1 with complete endothelial cell growth medium [DMEM supplemented with 10% fetal bovine serum, 1% Antibiotic-Antimycotic solution (Gibco, MA), 4 μg/ml endothelial cell growth supplement (Corning, 354006), 1% Non-essential amino acids (Gibco, MA), and 10 mM HEPES (Gibco, MA)]. The endothelial surface of each vessel was scraped into fibronectin-coated tissue culture dishes containing complete medium. Cultures were expanded and frozen at passage 1. The endothelial phenotype of the preparation was confirmed by evaluating cellular uptake of the endothelium-specific marker DiI-acetylated low-density lipoprotein. Experiments were conducted in cells obtained from three control and three diabetic male and four control and four diabetic female rats. The day before the experiments, cells were plated in commercial endothelial cell growth medium (ScienCell, CA) supplemented with 25 mM glucose (ScienCell, CA).

Assays for Oxidant Generation in Intact Cells

H_2O_2 generation was measured using Amplex Red and horseradish peroxidase (HRP) as previously described (Vázquez-Medina et al., 2016). Briefly, cells were incubated with 50 μM Amplex Red (Thermo Fisher, MA) and 2.5 U/ml HRP (Sigma) for 30 min at 37°C. The medium was collected, and absorbance was measured at 572 nm. At the end of the experiments, the cells were dissociated from the dishes. Protein content was measured by the BCA gold assay (Thermo Scientific, MA) and results were normalized to protein content. Intracellular oxidant generation was monitored by fluorescence microscopy using CellROX reagent (Thermo Scientific, MA). Cells loaded with 5 μM CellROX and NucBlue (Thermo Fisher, MA) were incubated for 30 min at 37°C. Cells were rinsed three times and imaged using an inverted fluorescence microscope (Zeiss Axio Observer) fitted with a 20× objective and Zen software. Fluorescence intensity in five fields per sample was quantified using ImageJ (NIH) and normalized to cell number.

Statistical Analysis

All values are expressed as mean ± standard error of the mean (SEM). Relaxation responses to each concentration of ACh and SNP were calculated as the percentage of relaxation from maximum PE contraction. Similarly, the recorded increase in the force of contraction was calculated as the percentage of maximum contraction obtained with PE at the highest dose or as changes in tension with increasing concentration of PE in the aortic rings. The concentration of agonist that produced half of the maximum effect (E_{max}) was expressed as EC_{50} and calculated by a sigmoidal dose–response model (for variable slope) using GraphPad Prism v7 (GraphPad Software Inc., San Diego, CA, United States). Sensitivity to each agonist was expressed as pD_2 values (−log [EC_{50}]), which were normally distributed. The area under the curve (AUC) was determined using GraphPad Prism 8 with trapezoidal technique. To compare the effect of different EDRFs such as COX, the ACh results were expressed as differences in the area under the concentration response curve (ΔAUC) between control (absence of Indo) and experimental (presence of Indo) conditions. One-way ANOVA was used to compare means among experimental groups (e.g., EC_{50}, E_{max}, and metabolic data). When the one-way ANOVA test returned $p < 0.05$, *post hoc* analyses were performed using Tukey's test. Comparison of CRCs between two groups was done using two-way ANOVA, with one factor being concentration and the other being group (male vs. female and control vs. diabetic). When the ANOVA test returned $p < 0.05$, *post hoc* analyses were performed using Bonferroni's or Tukey's test. Comparison of CRCs in a pre/post-test format within a group was done using two-way ANOVA with repeated measures. Three-way ANOVA with factors being sex, diabetes, and drugs (male vs. female, diabetic vs. non-diabetic and no drug vs. drug treatment) was used to compare group means. When the

ANOVA test returned $p < 0.05$, *post hoc* analyses were performed using Tukey's test.

For ROS generation assays, means were compared between control and diabetic groups using unpaired Student's *t*-tests for both males and females. Normality was confirmed using the Shapiro–Wilk test. Statistical analyses were conducted using GraphPad Prism v8.4.3. Values were considered different when $p < 0.05$. Student's unpaired *t*-test was used for comparisons of two group means (e.g., protein expression studies).

RESULTS

Metabolic Parameters and Insulin Signaling in UCD-T2DM Rats

Body weights of both male and female diabetic rats were significantly higher compared with the respective non-diabetic controls (**Table 1**). Accordingly, the weight of intra-abdominal adipose tissue, as well as its ratio to body weight (adiposity), was higher in diabetic rats than in non-diabetic control groups for both sexes. Moreover, male and female UCD-T2DM rats had higher triglyceride levels in plasma than did the respective non-diabetic controls. When compared to male UCD-T2DM rats, female UCD-T2DM rats had significantly higher circulating triglyceride levels and adiposity. Furthermore, both fasting glucose and HbA1c levels were higher in male and female diabetic rats compared to their respective non-diabetic controls. Fasting plasma insulin concentration was significantly higher in female diabetic rats compared with those in both the non-diabetic female control and male diabetic groups (**Table 1**). Similar to the previous report (Shaligram et al., 2020), there was no difference in plasma insulin levels in male diabetic rats when compared with their respective non-diabetic controls. However, the ISI was significantly lower in diabetic groups, regardless of sex, indicating that insulin signaling may be impaired in diabetic groups of both sexes. When compared to male UCD-T2DM rats, female UCD-T2DM rats had a lower ISI (**Table 1**).

The reduced ISI observed in the current study prompted us to analyze the expression of the main insulin signal transducers, insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2), in aortic tissue. As shown in **Figure 1A**, IRS-1 expression was significantly reduced in both male and female diabetic groups (by 0.5-fold and 0.6-fold in male and female diabetic groups, respectively). In contrast, only the female diabetic group displayed reduced IRS-2 protein expression (0.5-fold) compared to the sex-specific non-diabetic control (**Figure 1B**).

Relaxation Responses to ACh in UCD-T2DM Rat Aortas

A sex difference was observed in aortic relaxation responses to ACh in non-diabetic control rats. Both sensitivity, as assessed by $-\log [EC_{50}]$ (pD_2) value, and E_{max} to ACh were significantly higher in female than in male aortas (**Table 2**). In controls, the pD_2 to ACh was 6.57 ± 0.1 in male and 7.20 ± 0.1 in female aortas; the ACh E_{max} was $81.14\% \pm 1.6\%$ in male and $94.72\% \pm 1.9\%$ in female aortas (n = 6–12 per group, $p < 0.05$, one-way ANOVA).

When compared to the male non-diabetic control group, a potentiated relaxation response to ACh was observed in the male diabetic group (**Figure 2A**). Both the pD_2 and E_{max} of aortic rings to ACh were significantly enhanced in the male diabetic group (**Table 2**). The pD_2 to ACh was 6.57 ± 0.1 in control males and 7.14 ± 0.0 in diabetic males; the ACh E_{max} was $81.14\% \pm 1.6\%$ in control males and $94.04\% \pm 0.9\%$ in diabetic males (n = 11–12 per group, $p < 0.05$, one-way ANOVA). However, the E_{max} but not the sensitivity of aortic rings to ACh was reduced slightly but significantly in the female diabetic group compared to its respective control and the diabetic male group (**Figure 2B** and **Table 2**). The ACh E_{max} was $94.7\% \pm 1.9\%$ in control females and $85.9\% \pm 2.0\%$ in diabetic females (n = 6–7 per group, $p < 0.05$, one-way ANOVA).

Relative Contribution of EDRF to ACh-Induced Relaxation in UCD-T2DM Rat Aorta

The relative contributions of PGI_2, cGMP, and NO to vasorelaxation induced by ACh were estimated by sequentially inhibiting COX, sGC, and NOS. Specifically, EDV to ACh

TABLE 1 | Body weight and adipose weight, blood glucose levels, HbA1c, and other metabolic parameters of male and female control and diabetic rats.

	n	Male control	Male diabetic	Female control	Female diabetic
Body weight (g)	9–12	314.40 ± 21.7	529.60 ± 20.8*	210.47 ± 6.4#	403.99 ± 6.8*
Adipose tissue (g)	9–12	1.25 ± 0.1	11.77 ± 0.9*	1.56 ± 0.2	14.06 ± 1.8*#
Adipose tissue/body weight (g)	9–12	0.0043 ± 0.0	0.02 ± 0.0*	0.007 ± 0.0	0.036 ± 0.0*#
Triglyceride (mmol/L)	9–12	1.30 ± 0.1	1.94 ± 0.2*	1.27 ± 0.0	3.62 ± 0.4*#
Blood glucose (mg/dl)	9–12	132.25 ± 8.4	302.23 ± 29.3*	144.88 ± 12.5	388.73 ± 34.3*
HbA1c level	9–12	4.23 ± 0.1	10.95 ± 0.8*	4.43 ± 0.0	9.99 ± 0.9*
Insulin (ng/ml)	8–10	0.91 ± 0.5	0.96 ± 0.2	0.49 ± 0.1	5.71 ± 1.9*#
ISI	8–10	1.35 ± 0.1	0.60 ± 0.1*	0.89 ± 0.2	0.10 ± 0.0*#

Data are expressed as mean ± SEM.
n, number of rats per group.
**$p < 0.05$ (vs. control, same sex), #$p < 0.05$ (vs. male, respective group), using one-way ANOVA followed by Tukey's post hoc test.*
Insulin sensitivity index (ISI) = [2/(blood insulin (nM) × blood glucose (μM) + 1]. HbA1c = glycated hemoglobin (A1c).

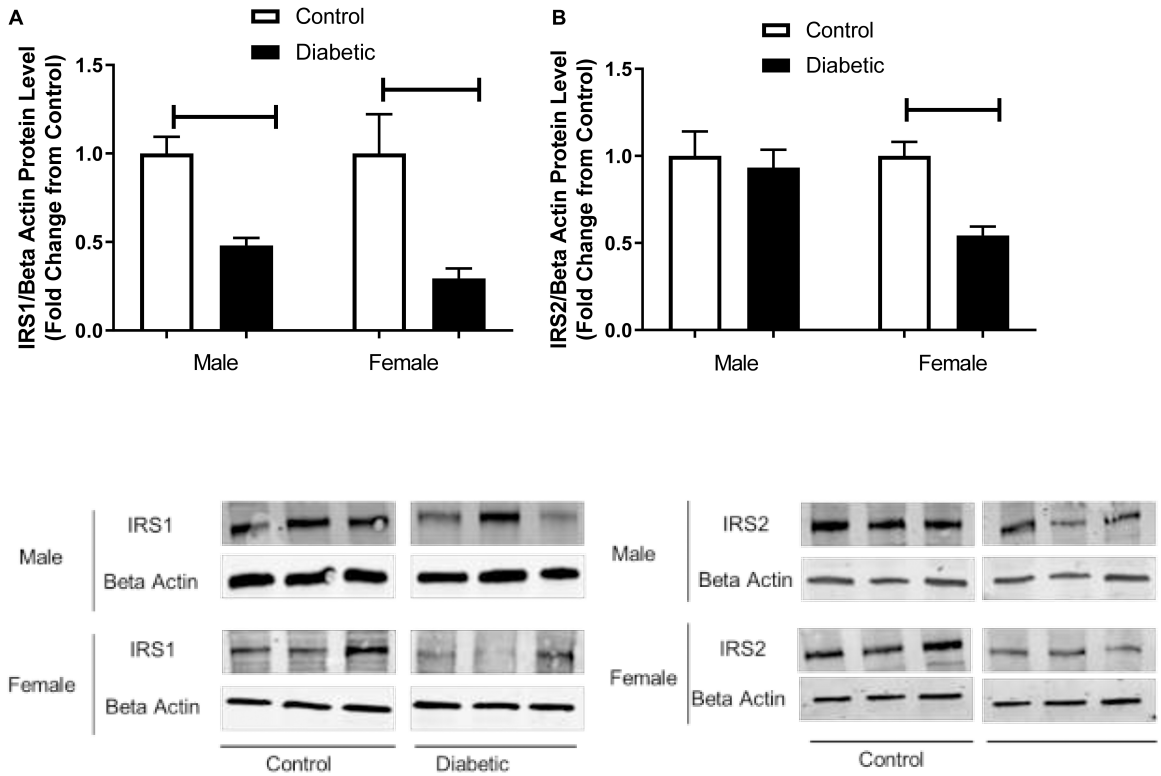

FIGURE 1 | Western blot analysis of IRS1 and IRS2 expression in control and UCD-T2DM rat aorta. Protein levels of **(A)** aortic insulin receptor substrate-I (IRS1) and **(B)** insulin receptor substrate-2 (IRS2) from the samples of male and female control and diabetic rats. IRS1 **(A)** and IRS2 **(B)** were quantified by densitometry and normalized to corresponding beta actin. Values are represented as mean ± SEM. Each bar represents the values obtained from n = 4–5 animal s per group. To show representative bands, images from different parts of the same gel have been juxtaposed, indicated by white dividing lines. Capped lines indicate significant differences between two groups ($p < 0.05$), as analyzed by unpaired Student 's *t*-test.

(10^{-8} to 10^{-5} M) in rat aortic rings pre-contracted with PE (2 μM) was obtained before and after pretreatment with Indo (10 μM), followed by the addition of ODQ (10 μM) and L-NNA (100 μM). When ODQ was added, the EDV reduction is thought to represent the impact of NO-dependent cGMP on EDV (Pieper and Siebeneich, 1997). Finally, addition of L-NNA, after inhibition of sGC by ODQ, represents the impact of NO independent of cGMP (Bolotina et al., 1994), and the slight remaining EDV to ACh is referred to as the L-NNA, Indo-insensitive component-type relaxation (Feletou and Vanhoutte, 1988).

TABLE 2 | pD_2 and E_{max} to acetylcholine (ACh) in aortic rings from male and female control and diabetic rats.

Ach	*n*	pD_2	E_{max} (%)
Male control	12	6.57 ± 0.1	81.14 ± 1.6
Male diabetic	11	7.14 ± 0.0*	94.04 ± 0.9*
Female control	6	7.20 ± 0.1#	94.72 ± 1.9#
Female diabetic	7	7.07 ± 0.1	85.92 ± 2.0*#

Data are expressed as mean ± SEM.
n, number of rats per group.
**p < 0.05 (vs. control, same sex), #p < 0.05 (vs. male, respective group), analyzed using one-way ANOVA followed by Tukey's post hoc test.*

The administration of Indo to block COX activity had no apparent effects on pD_2 and E_{max} to ACh, regardless of sex or diabetes status. The ΔAUC, defined as the difference in the AUC between the CRC to ACh before and after Indo, was not different between UCD-T2DM groups and respective non-diabetic control groups in either sex (**Figures 3A–D** and **Table 3**). Addition of ODQ completely blocked the remaining relaxation in all experimental groups except for the male diabetic group (**Figure 3B**). After adding ODQ, a slight but significant relaxation response remained in aortic rings of the male diabetic group compared to the control group (**Figure 3B** vs. **Figure 3A**). The E_{max} to ACh in male control and male diabetic aortas was 2.22% ± 0.2% and 10.14% ± 0.5%, respectively ($p < 0.05$, one-way ANOVA) (**Table 3**, third column). To examine whether the slight residual ACh-induced relaxation in male diabetic aortas may be due to the direct action of NO (independent of cGMP), a NOS inhibitor was used. The addition of L-NNA, a non-selective NOS inhibitor, had no apparent effect on the remaining Indo- and ODQ-resistant relaxation in male diabetic aortas (**Figure 3B**). After the addition of L-NNA, there was still a significant difference in the E_{max} to ACh between male control and male diabetic groups. The E_{max} to ACh was 0.43% ± 0.4% in male control and 13.38% ± 1.2% in male diabetic rats ($p < 0.05$, one-way ANOVA) (**Table 3**, fourth column). The remaining

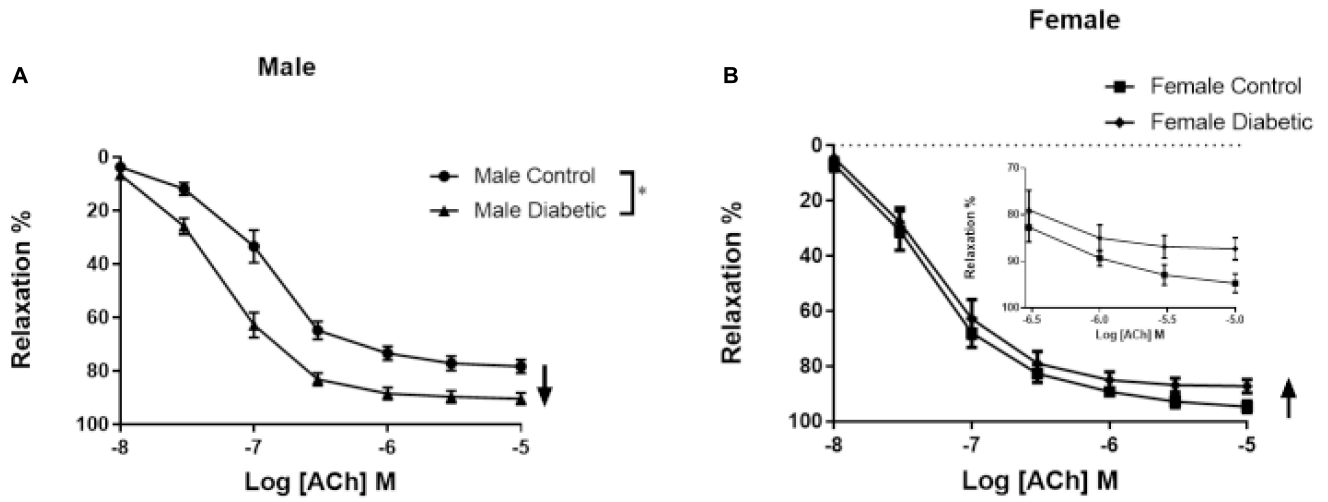

FIGURE 2 | Concentration-response curves to acetylcholine (ACh) in control and UCD-T2DM rat aorta. Relaxation responses to cumulative concentrations of ACh (10^{-8} to 10^{-5} M) in intact aortic rings pre-contracted with phenylephrine (PE, 2 μM) from male **(A)** and female **(B)** control and diabetic rats. Data are expressed as mean ± SEM. n = 6–12 animals per group. *$p < 0.05$ between two groups analyzed using two-way ANOVA followed by Bonferroni's post hoc test.

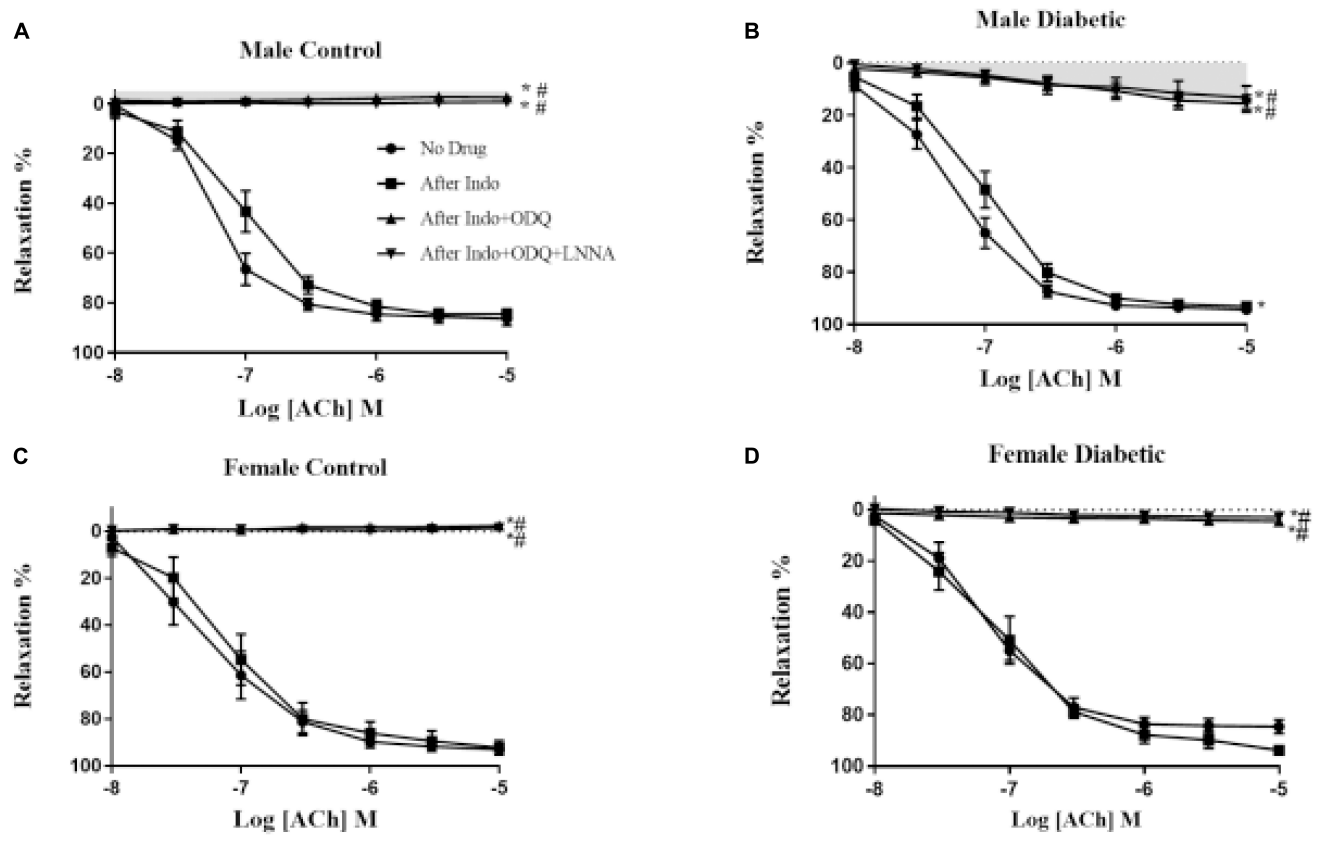

FIGURE 3 | Endothelium-derived relaxing factors (EDRF) contribution to acetylcholine (ACh)– induced relaxation responses in control and UCD-T2DM rat aorta. Effects of inhibiting cyclooxygenase, soluble guanylyl cyclase and nitric oxide synthase on ACh-induced vasorelaxation in aortic rings taken from **(A)** male control and **(B)** male diabetic, **(C)** female control and **(D)** female diabetic rats. ACh relaxation was measured in the presence of indomethacin (Indo, 10 μM), followed by addition of ODQ (10 μM), and then with N-nitro-L-arginine (L-NNA; 100 μM). Data are expressed as mean ± SEM. *$p < 0.05$ vs. no drug; $^{\#}P < 0.05$ vs. Indo; analyzed using two-way ANOVA with repeated measures followed by Bonferroni post hoc test (n = 5–8 per group). Light gray shaded area: contribution of endothelium-derived hyperpolarizing factor (EDHF)-type to endothelium-dependent vasorelaxation (EDV).

TABLE 3 | Area under the curve (ΔAUC), Sensitivity (pD_2: -logEC50) and maximum response (E_{max}) to ACh in rat aortic rings from male and female control and diabetic rats.

Groups	No drug			Indo			Indo + ODQ			Indo + ODQ + L-NNA		
	pD_2	E_{max} (%)	ΔAUC	pD_2	E_{max} (%)	ΔAUC	pD_2	E_{max} (%)	ΔAUC	pD_2	$E_{max\%}$	AUC
Male control	7.04 ± 0.1	83.82 ± 2.9	ND	6.72 ± 0.0	81.90 ± 2.31	28.2 ± 4.7	ND	2.22 ± 0.2^{ab}	163.99 ± 9.2	ND	0.43 ± 0.4^{ab}	2.79 ± 1.2
Male diabetic	7.18 ± 0.0	94.16 ± 1.1*	ND	6.95 ± 0.1	93.97 ± 0.3	23.7 ± 0.9	ND	10.14 ± 0.5*^{ab}	166.05 ± 5.1	ND	13.38 ± 1.2*^{ab}	11.15 ± 1.6*
Female control	7.31 ± 0.0	92.50 ± 0.7$^{\#}$	ND	7.29 ± 0.0	92.16 ± 2.8	6.35 ± 1.0	ND	2.38 ± 1.1^{ab}	185.60 ± 15.8	ND	1.51 ± 0.6^{ab}	3.28 ± 1.1
Female diabetic	7.19 ± 0.0	84.50 ± 2.7*$^{\#}$	ND	7.20 ± 0.0	93.78 ± 0.9	9.71 ± 4.6	ND	4.39 ± 0.6$^{ab\#}$	187.59 ± 13.5	ND	2.72 ± 0.7$^{ab\#}$	3.21 ± 1.2$^{\#}$

A comparison of the ΔAUC, sensitivity (pD_2), and maximum response (E_{max}) to acetylcholine in the absence (no drug) or in the presence of Indo, Indo + ODQ, and Indo + ODQ + L-NNA in aortic rings from male and female control and diabetic rats. Data are expressed as mean ± SEM.
$^{*}p < 0.05$ (vs. control, same sex), $^{\#}p < 0.05$ (vs. male in respective group) (one-way ANOVA followed by Tukey's post hoc test); $^{a}p < 0.05$ vs. no drug control within each group, $^{b}p < 0.05$ vs. indo within each group (two-way ANOVA with repeated measures followed by Tukey's post hoc test), n = 5–8 per group.
ND, not determined.

AUC after addition of L-NNA in the male diabetic group was significantly different from the male control group, suggesting a slight role of NO-PGI_2-independent relaxation responses in this group (**Table 3**, fourth column, **Figure 3B**, gray shaded area).

It is well known now that smooth muscle hyperpolarization results indirectly from the opening of endothelial SK_{Ca} and IK_{Ca} channels (McNeish et al., 2006). Therefore, an elevated NO-PGI_2-independent-type relaxation in aortas from the male diabetic group could be expected to result from significant overexpression of these hyperpolarizing K_{Ca} channels on the endothelium (Gillham et al., 2007). Next, Western blot analysis revealed that the expression of both SK_{Ca} and IK_{Ca} was significantly upregulated (by 9.0-fold and by 1.0-fold, respectively) in the aortic tissues from male diabetic rats compared with those in non-diabetic controls (**Figures 4A,B**).

Relaxation Responses to SNP in UCD-T2DM Rat Aorta

The smooth muscle sensitivity to NO was investigated by generating CRC to SNP (10^{-9} to 10^{-5} M) in endothelium-denuded aortic rings. No significant changes in either pD_2 values or E_{max} of SNP were observed in diabetic animals of either sex. The pD_2 values to SNP was 8.06 ± 0.0 in male control, 8.19 ± 0.1 in male diabetic, 8.50 ± 0.0 in female control, and 8.32 ± 0.0 in female diabetic animals. The E_{max} to SNP in male control and male diabetic animals was 100.15% ± 0.3% and 101.46% ± 1.4%, respectively. The E_{max} to SNP in female control and female diabetic animals was 104.34% ± 3.2% and 100.18% ± 0.4%, respectively.

Contractile Responses to PE in UCD-T2DM Rat Aorta

Contractile responses to an α-adrenoceptor agonist (PE) were analyzed by measuring the CRC to PE (10^{-8} to 10^{-5} M). There were no sex differences in PE contractile responses in aortic rings from non-diabetic control groups (**Figure 5**). However, both maximal tension developed in response to PE ($Tension_{max}$) and the sensitivity to PE in aortic rings were significantly enhanced in aortic rings of diabetic groups compared with the non-diabetic control rats, regardless of sex (**Figures 5A,B** and **Table 4**).

Next, the CRC to PE (10^{-8} to 10^{-5} M) was determined in aortic rings before and after pretreatment with the NO synthase inhibitor, L-NAME (200 μM), in the presence of Indo (10 μM). The changes in the contractile response to PE after the addition of L-NAME reveal the role of endothelium-derived NO during smooth muscle contraction in response to PE, as previously reported by us (Zhang et al., 2012; Sangüesa et al., 2017) and others (Hayashi et al., 1992; Dora et al., 2000).

The administration of Indo to block COX metabolites slightly but significantly reduced E_{max} to PE in aortas from male control and female diabetic groups, suggesting a slight elevation of the contractile metabolite of COX in this group, with no apparent effect on the maximal contractile response in female control and male diabetic groups (**Figure 6** and **Table 5**). The addition of L-NAME resulted in a significant increase of the contractile responses to PE in all experimental groups (**Figure 6**). However, ΔAUC (the difference in AUC between PE CRC before and after L-NAME) was lower in aortas of the diabetic rats compared with the control group, regardless of sex (**Table 5**).

Intracellular Pathways Related to Vascular Function in UCD-T2DM Rat Aortas

To investigate the mechanism by which endothelium-derived NO generation in response to PE might be reduced in diabetic animals, eNOS activation by phosphorylation was investigated by Western blot analysis. As shown in **Figure 7B**, the phosphorylation of eNOS at Ser-1177 was significantly reduced in aortic tissue from diabetic rats relative to controls, regardless of sex. Although the expression of total eNOS showed no significant difference between diabetic males and controls, its levels were significantly reduced in aortas from diabetic females (**Figure 7A**).

Vascular dysfunction could also be related to insulin resistance, and our results suggest that insulin signaling could be impaired in diabetic rats in both sexes. Next, we determined the expression of pAkt, which is downstream of IRS and an upstream mediator of eNOS phosphorylation at Ser-1177 in aortic tissues. **Figure 8B** shows that pAkt (Ser-473) content was significantly reduced in the aorta of diabetic rats compared with the control groups for both sexes, whereas total Akt protein

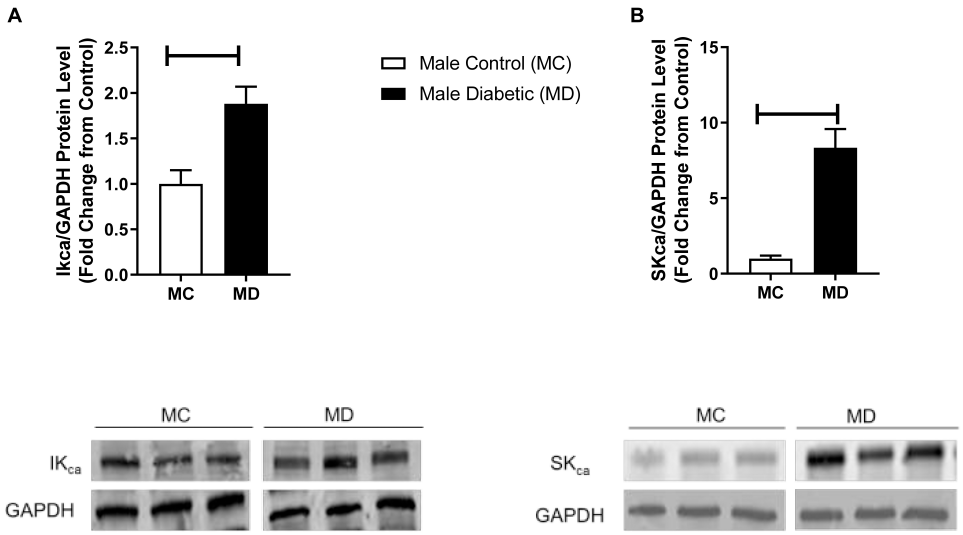

FIGURE 4 | Western blot analysis IK_{Ca} and SK_{Ca} expression in control and UCD-T2DM rat aorta. Protein levels of **(A)** aortic intermediate conductance calcium activated potassium channel (IK_{Ca}) and **(B)** small conductance calcium activated potassium channels (SK_{Ca}) were measured from the samples of male control and diabetic rats. IK_{Ca} **(A)** and SK_{Ca} **(B)** were quantified by densitometry and normalized to corresponding GAPDH. Values are represented as mean ± SEM. Each bar represents the values obtained from n = 4–5 per group. To show representative bands, images from different parts of the same gel have been juxtaposed, indicated by white dividing lines. Capped lines indicate significant differences between two groups ($p < 0.05$), as analyzed by unpaired Student's t-test. MC, male control; MD, male diabetic.

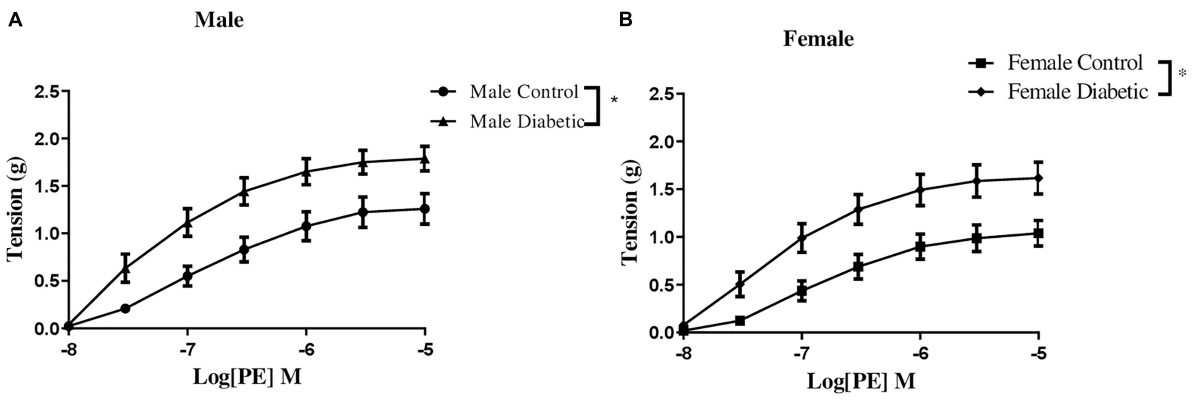

FIGURE 5 | Concentration-response curves to phenylephrine (PE) in control and UCD-T2DM rat aorta. Contractile responses to cumulative concentrations of PE (10^{-8} to 10^{-5} M) in intact aortic rings of **(A)** male and **(B)** female control and diabetic rats. Data are expressed as mean ± SEM. n = 5–6 per group. *$p < 0.05$ between two groups analyzed using two-way ANOVA followed by Bonferroni's *post hoc* test.

content remained unaffected by diabetes status, regardless of sex (**Figure 8A**).

To further investigate the possible mechanisms underlying the elevated responses to contractile agents in this model, the protein expression of NADPH oxidase (NOX) subtypes NOX1 and NOX4 was measured. NOX1 expression was significantly elevated in aortic tissues from diabetic groups, regardless of sex (1.5-fold in male diabetic and 1-fold in female diabetic rats, **Figure 9A**). However, NOX4 expression showed no significant differences among all experimental groups (**Figure 9B**).

Lastly, to examine whether the elevated expression of NOX1 in aortic tissues of diabetic rats was associated with elevated basal ROS levels in this group, intracellular and extracellular ROS generation was measured in primary endothelial cells isolated from aortic tissues using Amplex Red and CellROX assays. Both assays demonstrated that ROS generation was higher in the endothelial cells isolated from arteries of diabetic groups compared with those in the non-diabetic controls, regardless of sex (**Figures 9C,D**).

DISCUSSION

The present study demonstrates that aortic function in UCD-T2DM rats is altered in both sexes. It also provides the first

TABLE 4 | pD_2 and $Tension_{max}$ to phenylephrine (PE) in aortic rings from male and female control and diabetic rats.

PE	*n*	pD_2	$Tension_{max}$ (g)
Male control	5	6.81 ± 0.0	1.26 ± 0.1
Male diabetic	5	7.19 ± 0.0*	1.79 ± 0.1*
Female control	5	6.78 ± 0.0	1.04 ± 0.1
Female diabetic	6	7.14 ± 0.0*	1.53 ± 0.1*

Data are expressed as mean ± SEM.
n, number of rats per group.
**p < 0.05 (vs. control, same sex), analyzed using one-way ANOVA followed by Tukey's post hoc test.*

evidence of sexual dimorphism in aortic relaxation in UCD-T2DM rats.

In the current study, while both male and female diabetic rats had higher body weight and hyperglycemia compared with non-diabetic control rats, the female diabetic group exhibited higher adiposity, triglyceride, and insulin levels than control or male diabetic rats. This is consistent with the results from our previous study (Shaligram et al., 2020). Similarly, Ohta et al. (2014) which reported elevated blood insulin levels in spontaneously diabetic torii (SDT) female rats compared with SDT male rats. In the current study, the ISI was lowered in diabetic groups, irrespective of sex. However, when compared to male diabetic rats, female diabetic rats exhibited a lower ISI. Accordingly, here, we showed that insulin signaling was impaired in the aortic tissues in diabetic groups in both sexes. Notably, aortic IRS-1 was reduced to a similar extent in both diabetic groups, but IRS-2 was reduced only in the female diabetic group. It has been reported that the downregulation of IRS-2 levels in endothelial cells is induced by hyperinsulinemia in obese subjects (Kubota et al., 2011). Similarly, our results on elevated insulin levels in female diabetic rats may suggest that the decreased

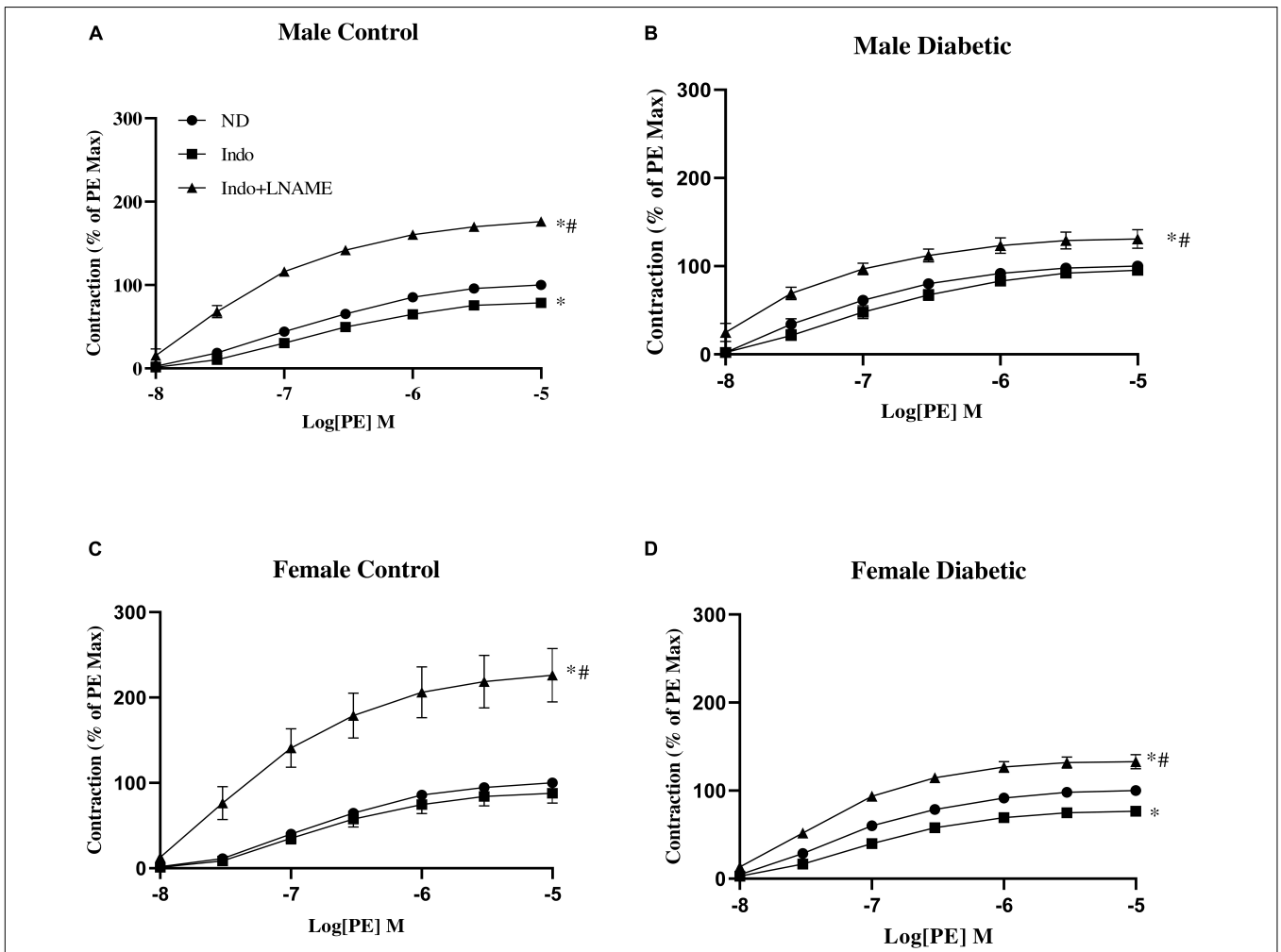

FIGURE 6 | Concentration-response curves to phenylephrine (PE) in intact aortic rings from **(A)** male control, **(B)** male diabetic, **(C)** female control, and **(D)** female diabetic rats. Contraction to PE was measured in absence of any drug (ND) or in presence of indomethacin (Indo, 10 μM) followed by addition of *N*-Nitro-L-arginine methyl ester (Indo + L-NAME, 200 μM). Results are expressed as a percent of the maximal response to PE (10 μM) obtained in the absence of any drug. Data are expressed as mean ± SEM, analyzed using two-way ANOVA with repeated measures followed by Tukey's *post hoc* test: $^{*}p < 0.05$ vs. ND; $^{\#}p < 0.05$ vs. Indo, n = 6–8 per group.

TABLE 5 | E_{max}, $Tension_{max}$, pD_2, and ΔAUC to phenylephrine (PE) in aortic rings from male and female control and diabetic rats.

	$E_{max(\%)}$	$Tension_{max}$ (g)	pD_2	ΔAUC
Male control (*n* = 6)				
ND	100	1.26 ± 0.1	6.81 ± 0.0	
Indo	78.72 ± 4.8[a]	1.09 ± 0.1	6.74 ± 0.0	239.10 ± 5.0
Indo + L-NAME	176.15 ± 3.6[b]	2.47 ± 0.2[b]	7.29 ± 0.0[b]	
Male diabetic (*n* = 8)				
ND	100	1.79 ± 0.1[c]	7.19 ± 0.0[c]	
Indo	95.39 ± 5.5	1.66 ± 0.1	6.95 ± 0.1	103.66 ± 18.1*
Indo + L-NAME	132.58 ± 8.7[bc]	2.26 ± 0.1[b]	7.46 ± 0.0[b]	
Female control (*n* = 6)				
ND	100	1.04 ± 0.1	6.78 ± 0.0	
Indo	87.83 ± 11.6	0.93 ± 0.1	6.76 ± 0.0	319.44 ± 59.8
Indo + L-NAME	226.19 ± 31.2[b]	2.25 ± 0.2[b]	7.21 ± 0.1[b]	
Female diabetic (*n* = 7)				
ND	100	1.53 ± 0.1[c]	7.14 ± 0.0[c]	
Indo	78.94 ± 2.2[a]	1.19 ± 0.1	7.01 ± 0.0	167.42 ± 6.8*
Indo + L-NAME	134.23 ± 9.4[bc]	1.98 ± 0.1[b]	7.35 ± 0.0[b]	

Data are expressed as mean ± SEM. Three-way ANOVA with factors being sex, diabetes, and drugs were used to compare among group means of E_{max} (%), $Tension_{max}$, and pD_2.

[a]$p < 0.05$ vs. (ND), [b]$p < 0.05$ vs. (Indo); [c]$p < 0.05$ (vs. control, same sex); analyzed using three-way ANOVA followed by Tukey's post hoc test.

ΔAUC, differences of area under the concentration–response curve with or without L-NAME in the presence of indo.

**$p < 0.05$ (vs. control, same sex), analyzed using two-way ANOVA followed by Tukey's post hoc test. n = 6–8 per group.*

IRS-2 expression observed in the aorta could be in part due to hyperinsulinemia in this group.

In T2D, impaired (Sakamoto et al., 1998), enhanced (Zhong et al., 2012), or preserved (Bohlen and Lash, 1995) EDV has been reported. Here, a slight but significant decrease in maximum relaxation to ACh was observed in aortic rings from female UCD-T2DM rats compared to their respective controls. However, an intriguing observation of this study was that aortic rings from male diabetic animals exhibited a potentiation in EDV compared with that in male controls. Similar observations were also made by our group using Zucker diabetic fatty (ZDF) male rats. Specifically, obesity-induced diabetes (ZDF model) significantly impaired relaxation responses to ACh in aortic rings taken from females, but potentiated the relaxation in males (data not shown). In accordance with our current study, Zhong et al. (2012) reported elevated relaxation responses to ACh in aortic rings of GK male rats. On the other hand, Kazuyama et al. (2009) and Nemoto et al. (2011) reported an impaired EDV in aortic rings from GK male rats.

It has been well established that in conduit arteries, NO plays a major role in EDV (Shimokawa et al., 1996; Gao et al., 2011). The impaired EDV may result from either a decreased NO production or an increased inactivation of NO by ROS. It has been reported that T2D reduces the synthesis of NO in rat aorta by phosphorylation of eNOS at Ser-1177 (Nemoto et al., 2011). Here, we did not directly measure NO production, but our data show that the expression of the active, phosphorylated form of eNOS is decreased while the expression of NOX1 and ROS generation are increased in aortas from diabetic groups in both sexes, suggesting that decreased NO bioavailability may in part contribute to reduced responses to ACh in diabetic female arteries. However, elevated ACh responses in diabetic male aorta cannot be attributed to decreased NO due to decreased eNOS activation or elevation of ROS, suggesting that other factors may be involved.

There is an established negative regulatory effect of NO on EDHF synthesis (Bauersachs et al., 1996; Brandes et al., 2000), and an augmented EDHF response was also shown to compensate for the loss of NO in arteries in diabetic rats (Garland et al., 1995; Malakul et al., 2008). In agreement with those studies that demonstrate compensatory interactions between pathways, the potentiation of the ACh response (regardless of decreased eNOS activity) in aortic rings from the male diabetic group suggests that other vasodilatory molecules besides NO may be involved in ACh relaxation in this group.

In the present study, we showed that the inhibition of COX metabolites by Indo did not alter relaxation responses to ACh significantly in aortic rings of any of the four experimental groups. Consistent with these results, Malakul et al. (2008) reported that hypercholesterolemia and type 1 diabetes did not have any effect on COX-mediated EDV in rat aortas. Here, addition of ODQ completely abolished the EDV in aortic rings of control groups of both sexes as well as female, but not male, diabetic groups, suggesting that EDV is solely mediated by NO acting on the sGC (NO-dependent cGMP) pathway in the above-mentioned groups. Although the vasodilatory effect of NO on vascular smooth muscle is mainly mediated by cGMP (*via* a cGMP-dependent K^+-channel activation) (Taylor et al., 2001), a direct effect of NO on Ca^{2+}-dependent K^+-channels (Bolotina et al., 1994) and L-type calcium current (Summers et al., 1999) without requiring cGMP has also been demonstrated. Here, the slight remaining Indo-ODQ-resistant relaxation in the aorta of male diabetic rats was unaffected by L-NNA, suggesting that NO-independent cGMP does not play a role in EDV in this group. An alternative explanation for the relaxation resistance to sGC and NOS inhibition in male diabetic aortas may be the contribution of other factors (NO- and PGI_2-independent) on relaxation in this group. Similarly, Malakul et al. (2008) reported a potential role of EDHF in EDV in aortas of streptozotocin-induced type 1 diabetic male rats. However, they did not include females in their studies to determine whether there was a sex effect in the type 1 diabetic rat aorta. There are also other reports of a decreased NO-dependent relaxation response and increased EDHF activity in saphenous arteries (Chadha et al., 2010) and carotid arteries (Leo et al., 2010) of high-fat diet-induced obese and type 1 diabetic male rats, respectively.

Epidemiological studies suggest that males are at higher risk for CVD compared to age-matched females during their reproductive years (Liu et al., 2003). This sex difference has been attributed to estrogen's protective effect in females and/or a detrimental androgen effect in males (Thompson and Khalil, 2003). However, a growing body of evidence suggests that androgens exhibit protective actions on the cardiovascular system (Nettleship et al., 2009). Administration of testosterone has been shown to induce both endothelium-dependent and independent vasorelaxation in rabbit aorta (Yue et al., 1995),

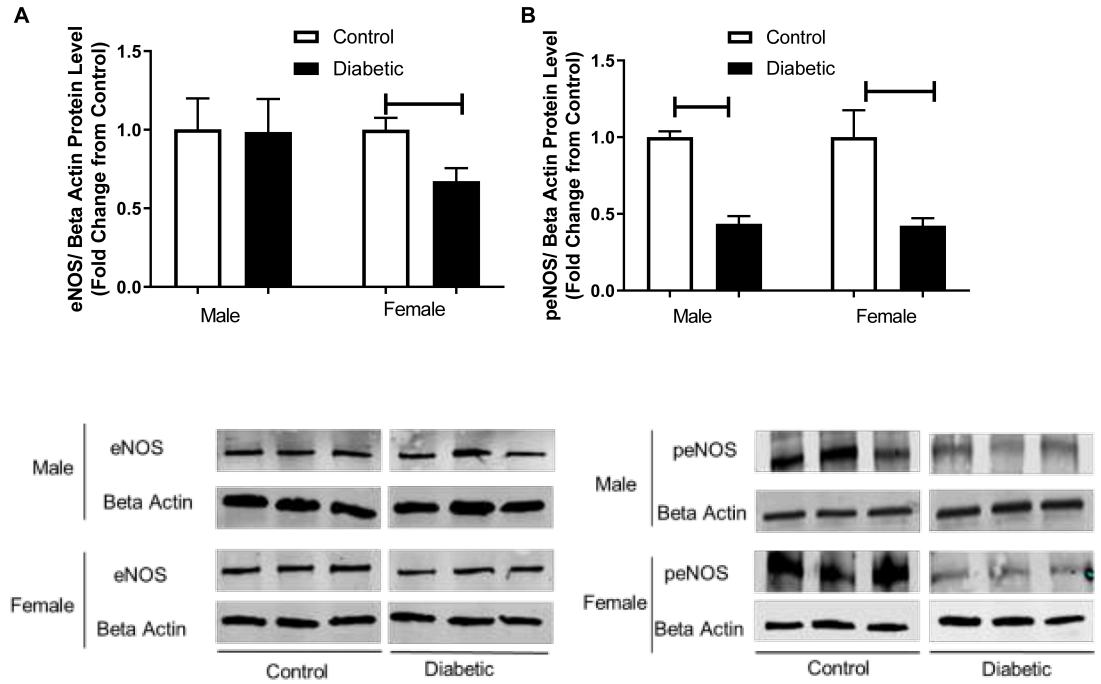

FIGURE 7 | Western blot analysis of eNOS and peNOS expression in control and UCD-T2DM rat aorta. Protein levels of **(A)** total endothelial nitric oxide synthase (eNOS) and **(B)** phosphorylated eNOS (peNOS) in aortic samples from male and female control and diabetic rats. eNOS **(A)** and peNOS **(B)** were quantified by densitometry and normalized to corresponding beta actin. Each bar represents the mean ± SEM of values obtained from n = 4–5 animals per group. To show representative bands, images from different parts of the same gel have been juxtaposed, indicated by white dividing lines. Capped lines indicate significant differences between two groups ($p < 0.05$), as analyzed by unpaired Student's t-test.

rat aorta (Costarella et al., 1996), and porcine coronary artery (Crews and Khalil, 1999).

Here, our data show that control female aortas exhibit greater ACh-mediated vasorelaxation compared to male aortas. This is in accordance with our previous report on the sex difference in rat aortic relaxation (Rahimian et al., 1997); however, we have now extended these findings in reporting that under the T2D condition, beneficial effects of female hormones could be lost, yet, intriguingly, male aortas exhibit greater ACh-mediated relaxation.

The K_{Ca} currents are mainly mediated by IK_{Ca} and SK_{Ca} channels (Brähler et al., 2009) in conduit and resistance-sized arteries in many species, including humans (Taylor et al., 2001; Félétou, 2009; Grgic et al., 2009). Taylor et al. (2001) reported that NO-independent relaxations evoked by ACh in rabbit conduit arteries were sensitive to a combination of SK_{Ca} and IK_{Ca} channel blockers. Although the effects of SK_{Ca} and IK_{Ca} channel blockers were not examined in the current study design, our data on the significant increase in expression of IK_{Ca} and SK_{Ca} channels in male diabetic arteries suggest that the slight NO- and PGI_2-independent relaxation observed in this group may be associated with these channels. In a preliminary functional study, further examination of IK_{Ca} and SK_{Ca}, using selective inhibitors of these channels, suggested a role for IK_{Ca} in ACh-induced relaxation in aorta of male diabetic rats (data not shown). These results are also in accordance with Schach et al. (2014) who reported an elevation of expression and contribution of IK_{Ca} in mesenteric arteries of male ZDF rats. In the current study, we observed no significant differences in the expression of IK_{Ca} channels in aorta from female diabetic compared with female control animals (n = 5–6, data not shown). It is also important to note that in aorta from males, the contribution of a NO-independent factor to the ACh response was only observed in the diabetic state and not in the control (or healthy) state. Sandow et al. (2009) reported that EDHF is present in aortas of juvenile rats (Martínez-Orgado et al., 1998) and disease models (such as hypercholesterolemic, diabetic, hypertensive, and with altered estrogen levels), but absent in healthy adult aorta (Kagota et al., 2000; Matsumoto et al., 2004; Woodman and Boujaoude, 2004; Malakul et al., 2008).

Besides the possibility of a modified contribution of NO, alteration of EDV to ACh in aortas of the UCD-T2DM model could be explained by changes in smooth muscle responsiveness to NO or contractile agents. However, our data showed that SNP (a NO donor)-induced relaxation of endothelium-denuded aortic rings was not altered in male or female diabetic groups. Similarly, Nemoto et al. (2011) observed no significant difference in SNP-induced relaxation in aortic rings of male GK rats. This suggests that smooth muscle responsiveness to NO in the aorta was not affected in UCD-T2DM rats. On the other hand, in the current study, the sensitivity and maximum tension to PE were enhanced significantly in aortic rings of UCD-T2DM groups compared with their respective controls, regardless of sex (**Figure 5**). The elevated PE response may in part explain the slight but significant decrease in the maximum relaxation in ACh

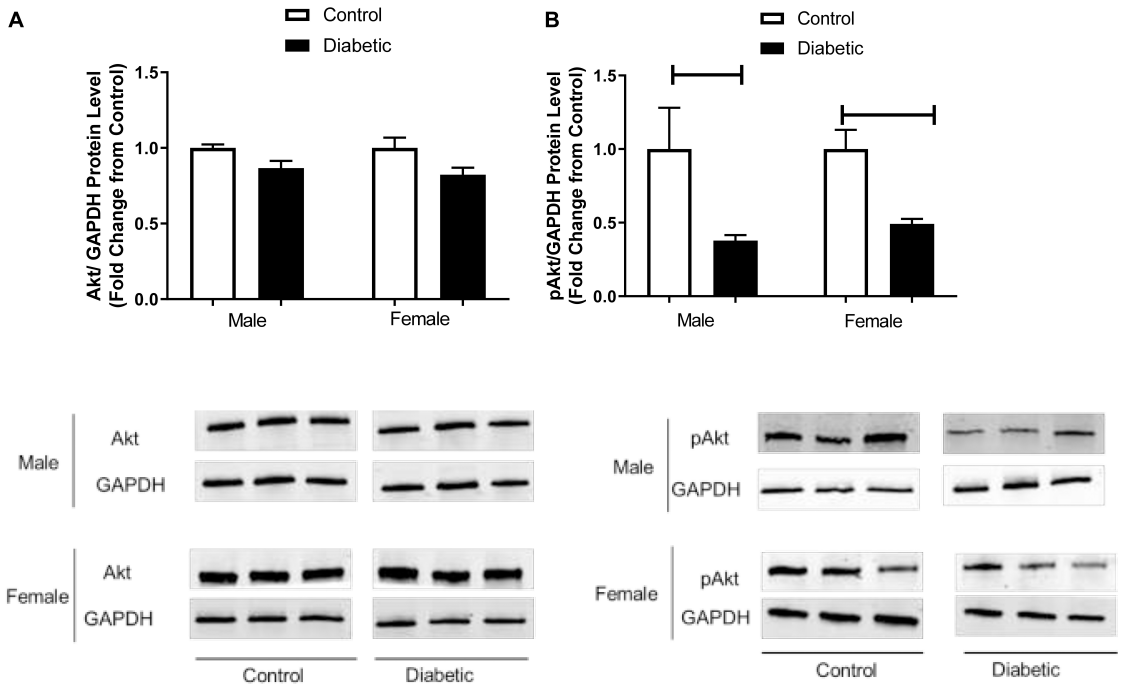

FIGURE 8 | Western blot analysis of Akt and pAkt expression in control and UCD-T2DM rat aorta. Protein levels of aortic **(A)** total V-akt murine thymoma viral oncogene homolog-2 (Akt) and **(B)** phosphorylated V-akt murine thymoma viral oncogene homolog-2 (pAkt) from the samples of male and female control and diabetic rats. Akt **(A)** and pAkt **(B)** were quantified by densitometry and normalized to corresponding GAPDH. Each bar represents the mean ± SEM of values obtained from $n = 4$–5 animals per group. To show representative bands, images from different parts of the same gel have been juxtaposed, indicated by white dividing lines. Capped lines indicate significant differences between two groups ($p < 0.05$), as analyzed by unpaired Student's *t*-test.

responses in aorta of female diabetic rats. However, it is important to note that regardless of increased PE-induced contraction, the ACh response was enhanced in male diabetic arteries. This, therefore, excludes the diminished PE contractile responsiveness as the cause of the increased ACh responses observed in male diabetic arteries. Nevertheless, our data on PE responses are in line with previous findings that type 1 diabetes results in increased vascular contraction in rat aortas (Abebe et al., 1990) and mesenteric arteries (White and Carrier, 1990).

Phenylephrine may indirectly stimulate endothelial cells to release NO *via* a signal transmitted either through myoendothelial gap junctions (Dora et al., 2000; Jackson et al., 2008) or by mechanical stress (Fleming et al., 1999). Therefore, the elevated contractile responses to PE observed in UCD-T2DM male and female rats may in part result from a decreased release of NO from the endothelium during smooth muscle contraction or an enhanced release of contracting factors (Zhang et al., 2012).

In the current study, we assessed the role of endothelium-derived NO by measuring the difference in the degree of PE-induced contraction in the absence and presence of L-NAME (Hayashi et al., 1992; Han et al., 2016). Pretreatment with L-NAME caused a significantly lower potentiation of the PE response in aortic rings from UCD-T2DM rats, regardless of sex (**Figures 6B,D**) compared with their controls. This suggests that decreased basal NO activity may in part be responsible for the elevated PE contractile responsiveness in UCD-T2DM rats in both sexes. Here, our study was limited in that we did not directly measure basal NO level. Nevertheless, consistent with an important role for eNOS phosphorylation on serine 1177 by Akt in regulating basal NO release (Scotland et al., 2002; Kobayashi et al., 2004), a reduction in eNOS expression by phosphorylation at Ser-1177 was observed in aortas from diabetic rats in both sexes compared with their controls. Additional studies will be needed to document the direction and magnitude of these interactions along with the relative importance of NO to elevated contractile responses in UCD-T2DM male and female rats.

Insulin resistance is a key element in the pathogenesis of T2D (Ormazabal et al., 2018). Insulin resistance is associated with endothelial dysfunction by several mechanisms including increased production of pro-inflammatory vasoconstrictor factors and oxidative stress (Schneider et al., 2000; Del Turco et al., 2011). Previous studies on experimental models of insulin resistance revealed impaired insulin-mediated PI3K/Akt-dependent signaling in the vasculature (Jiang et al., 1999). Our data on the significant decrease in expression of IRS and pAkt (a downstream mediator of IRS and upstream of eNOS phosphorylation at Ser-1177) in aortic tissues of diabetic animals in both sexes, suggest that the decreased peNOS levels in the diabetic aorta could arise from altered activation by pAkt.

Finally, it has also been reported that in diabetes, oxidative stress and superoxide radical production derived from

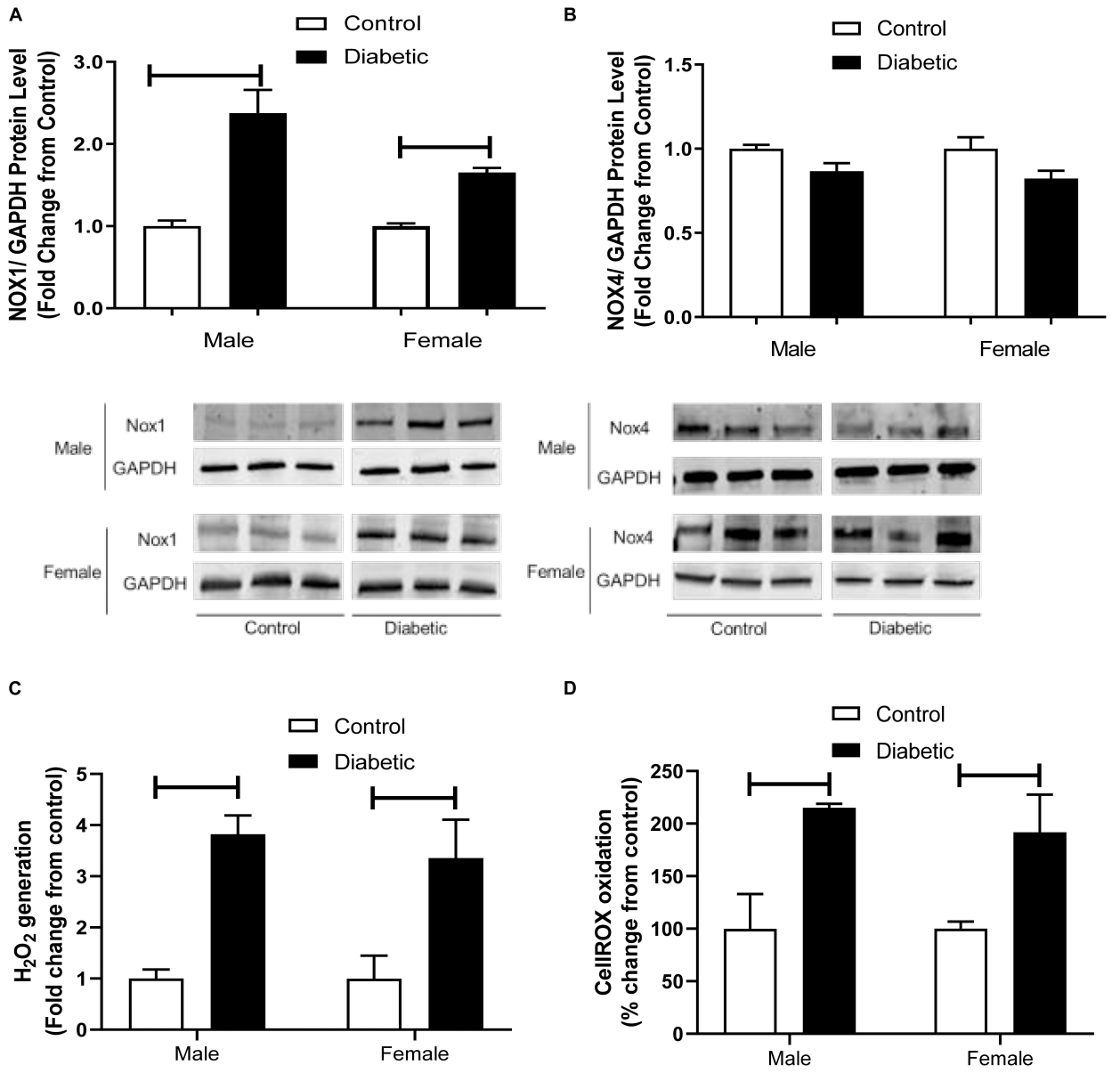

FIGURE 9 | Western blot analysis of NOX1 and NOX4 expression in intact aorta, and oxidant generation in primary aortic endothelial cells isolated from control and UCD-T2DM rats. Protein levels of aortic NADPH oxidases (NOX1) **(A)** and NOX4 **(B)** from the samples of male and female control and diabetic rats. NOX1 and NOX4 were quantified by densitometry and normalized to corresponding GAPDH. To show representative bands, images from different parts of the same gel have been juxtaposed, indicated by white dividing lines. Hydrogen peroxide (H_20_2) **(C)** and intracellular oxidant (CellROX oxidation) **(D)** generation in primary aortic endothelial cells isolated from male and female control and diabetic rats. Values are presented as mean ± SEM. Each bar represents the values obtained from n = 4–5 animals per group for NOX expression and n = 3–4 animals per group for oxidant generation studies. Capped lines indicate significant differences between two groups ($p < 0.05$), analyzed by unpaired Student's t-test.

insulin resistance may play a crucial role in enhancing the contracting responses (Shi et al., 2007). Superoxide scavenges NO, decreasing its bioavailability (Rubanyi et al., 1986), and elevating endothelium-dependent contractions. In the present study, we determined expression of NOX proteins, a source of superoxide. Vascular walls express high levels of NOX1, NOX2, and NOX4 (Griendling et al., 2000). NOX1 is mainly expressed in large conduit vessels (Lassègue et al., 2001), whereas NOX2 is more highly expressed in resistance vessels (Touyz et al., 2002). Here, we observed an elevated expression of NOX1 in aorta from diabetic groups, irrespective of sex, whereas NOX4 expression was not changed. Youn et al. (2012) reported that the activation of NOX1 was associated with eNOS uncoupling and endothelial dysfunction in streptozotocin-induced type 1 diabetic mice aorta. Furthermore, Gray et al. (2013) reported that genetic deletion of NOX1 in diabetic mice led to reduced

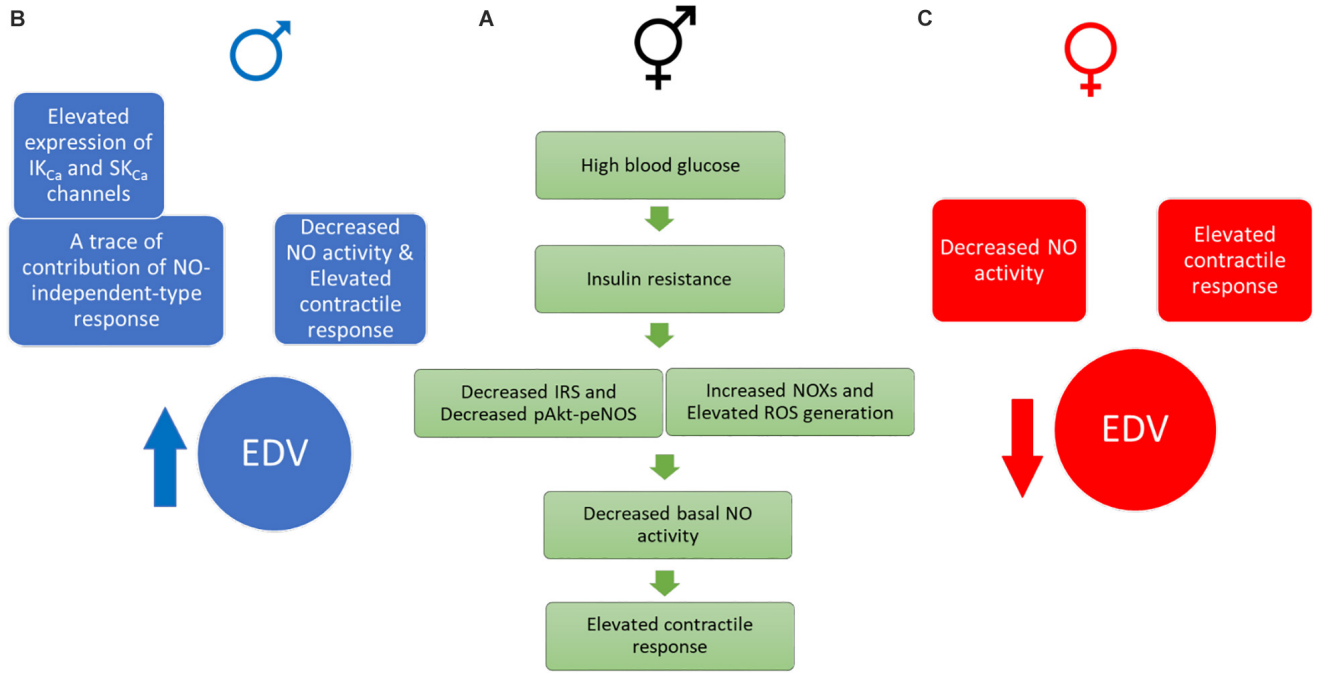

FIGURE 10 | Proposed drivers of an elevated contractile response in UCD-T2DM rat aortas. (**A** green one) Impaired insulin signaling, elevated ROS generation, and decreased basal NO activity may drive elevated contractile responses in aortic rings of both male and female UCD-T2DM rats. (**B** blue one) Male UCD-T2DM rat aortas display enhanced EDV (despite elevated contractile responses) along with elevated IK_{Ca} and SK_{Ca} channel expression and traces of NO-independent responses. (**C** red one) Female UCD-T2DM rat aortas display impaired EDV, possibly due to decreased NO activity and enhanced contractile responses. IRS, insulin receptor substrate; pAkt, phosphorylated V-Akt murine thymoma viral oncogene homolog-2; peNOS, phosphorylated endothelial nitric oxide synthase; NOX, NADPH oxidase; ROS, reactive oxygen species; NO, nitric oxide; IKCa, intermediate-conductance calcium- activated potassium channel; SKca, small-conductance calcium-activated potassium channel; EDV, endothelium-dependent vasorelaxation.

diabetes mellitus symptoms, suggesting a key role of NOX1-derived ROS in diabetes. Consistent with these results is the observation that ROS generation in aortic primary endothelial cells isolated from diabetic rats was higher than in cells isolated from control animals, regardless of sex. Taken together, our results on NOX1 upregulation and increased ROS generation in diabetic arteries suggest that the elevation of responses to PE observed in diabetic animals in both sexes may be partially due to the reduced NO bioavailability or increased in generation of potential vasoconstrictor substances (such as superoxide anions).

CONCLUSION

This study represents the first report showing that the aortic function in UCD-T2DM rats is altered in both sexes. Our data suggest that decreased insulin sensitivity, possibly *via* pAkt-dependent signaling and enhanced oxidative stress, may contribute to the elevated contractile responses in aorta of this model of T2D, regardless of sex. We also showed sex differences in aortic relaxation in this model. Specifically, our data show that under the T2D condition, beneficial effects of female hormones could be lost, yet, intriguingly, male aortas exhibit greater ACh-mediated relaxation. Additional studies will be needed to identify an underlying mechanism for the sex-specific differences observed in the aortic relaxation in this model.

Figure 10 depicts our proposed scheme based on the data presented in this report. Briefly, the elevation of contractile responses in aortic rings of both male and female UCD-T2DM could result from decreased basal NO activity, possibly due to the impaired insulin-mediated pAkt-peNOS dependent signaling and/or increased oxidative stress in this model (A). In the meantime, aortic vasorelaxation was elevated in aortic rings from male UCD-T2DM rats (B), but slightly impaired in female UCD-T2DM rats (C). The elevated vasorelaxation response in aortic rings from male diabetic rats (despite elevated contractile responses in this group) was accompanied by the elevated IK_{Ca} and SK_{Ca} channel expression and trace a of NO-independent responses (B). However, the decreased relaxation in female diabetic aortas could be in part attributed to the decreased NO activity and elevated contractile responses in this group (C).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of the Pacific and the University of California, Davis Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

FA designed and performed the majority of the experiments and drafted the manuscript. MR and SS assisted with the animal experiments. PH, KS, and JG generated the UCD-T2DM rat models and provided the intellectual input and critical reading of the manuscript. JV-M and KA performed the ROS experiments and provided the intellectual input. KA was also involved in editing the manuscript. RR was involved with the conception and design of research, revising the manuscript, and approving the final draft of manuscript. All the authors contributed to the article and approved the submitted version.

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Sex Differences and Regulatory Actions of Estrogen in Cardiovascular System

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Great progress has been made in the understanding of the pathophysiology of cardiovascular diseases (CVDs), and this has improved the prevention and prognosis of CVDs. However, while sex differences in CVDs have been well documented and studied for decades, their full extent remains unclear. Results of the latest clinical studies provide strong evidence of sex differences in the efficacy of drug treatment for heart failure, thereby possibly providing new mechanistic insights into sex differences in CVDs. In this review, we discuss the significance of sex differences, as rediscovered by recent studies, in the pathogenesis of CVDs. First, we provide an overview of the results of clinical trials to date regarding sex differences and hormone replacement therapy. Then, we discuss the role of sex differences in the maintenance and disruption of cardiovascular tissue homeostasis.

Keywords: cardiovascular disease, estrogen, sex hormones, cardiovascular homeostasis, non-nuclear signaling

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INTRODUCTION

Despite recent advances in medical and interventional therapies, cardiovascular disease (CVD) remains a leading global cause of death in men and women. Although sex differences are well recognized in the epidemiology and outcomes of CVD, their full extent is yet unclear. The results of recently published clinical studies on sex differences may provide new insights into the underlining mechanisms. A recent study that investigated the effect of sacubitril–valsartan on the incidences of cardiovascular death and hospitalization by heart failure (HF) in patients with HF with preserved ejection fraction (HFpEF) reported significantly reduced outcomes in women with HFpEF, but no statistically significant effect was observed in men with HFpEF (Solomon et al., 2019; McMurray et al., 2020). Sacubitril upregulates natriuretic peptide signaling of which cyclic guanosine monophosphate (cGMP) is considered the downstream target (Emdin et al., 2020). Intriguingly, sildenafil, another activator of cGMP signaling, via inhibition of phosphodiesterase type 5 (PDE5), showed sex differences in its beneficial effect on HF in animal models (Takimoto et al., 2005; Sasaki et al., 2014). Studies also showed that women with premature

Abbreviations: ARB, Angiotensin receptor blockers; CHIP, Clonal hematopoiesis of intermediate potential; CVD, Cardiovascular diseases; DOPS, Danish Osteoporosis Prevention Study; EC, Endothelial cells; HF, Heart failure; HFpEFHF, With preserved ejection fraction; HFrEFHF, With reduced ejection fraction; HT, Hormone therapy; IHD, Ischemic heart disease; VSMC, Vascular smooth muscle cells; WHI, Women's Health Initiative.

menopause more frequently embrace clonal hematopoiesis of intermediate potential (CHIP), the age-related expansion of hematopoietic stem cells with leukemogenic mutations without detectable malignancy, which is associated with the development of CVD (Jaiswal et al., 2017; Honigberg et al., 2021). Taken together, these clinical and experimental findings suggest clear sex differences in cardiovascular morbidity, natural course and drug efficacy.

The role of sex hormones in the development of CVD, particularly the effect of estrogen on the cardiovascular system, is strongly suggested as the cause of these sex differences. Indeed, several clinical trials, including recent large-scale clinical trials and many basic experiments, have shown the cardiovascular protective effects of estrogen (Bernelot Moens et al., 2012; Schierbeck et al., 2012; Hodis et al., 2016). However, some previous large-scale clinical trials have reported adverse effects of estrogen (Manson et al., 2003; Turgeon et al., 2004), so it seems estrogen may not be entirely beneficial. For clarity in this area, it is necessary to determine the mechanisms of action of estrogen in greater detail. Therefore, in this paper, we first outline the results of clinical trials to date that evaluated the preventive effects of estrogen against CVD, and then, we focus on the molecular function of estrogen signaling in terms of receptors, cell types, organs and pathological models. Finally, we discuss the mechanisms by which estrogen signaling elicits sex differences in the cardiovascular system.

SEX DIFFERENCES AND ESTROGEN HORMONE THERAPY IN CARDIOVASCULAR DISEASES

Sex Differences in Cardiovascular Diseases

Studies over the decades have reported a distinct pattern of CVD prevalence based on sex. Further, the latest epidemiological report stated that younger women have a lower risk of developing CVD, that the difference between sexes disappears at ages 60–79, and that women overtake men at the age of 80 (Virani et al., 2020), i.e., young premenopausal women have protection against CVDs, and the protection fades away after menopause. Therefore, the cardioprotective role of the female hormone estrogen has been regarded as a major factor responsible for the sex difference in the incidence of CVDs (Vitale et al., 2009).

The overall lifetime risk of HF is similar between the sexes, but sex differences in the epidemiology of HF become apparent when the type of HF is considered. HF with reduced left ventricular ejection fraction (HFrEF) is more common in men than in women (Lee et al., 2009; Dunlay et al., 2017). This type of HF is caused by previous myocardial infarction or dilated cardiomyopathy, and these two diseases are more prevalent in men than in women. In contrast, as revealed by the Framingham heart study, HFpEF is two times more common in women than in men (Lee et al., 2009; Dunlay et al., 2017). Given the fundamental differences in pathophysiology, HFpEF and HFrEF are managed differently. Although results of clinical trials on HFrEF demonstrate the effectiveness of beta blockers, angiotensin converting enzyme inhibitors, angiotensin receptor blockers (ARBs) and sodium–glucose cotransporter-2 inhibitors, these therapies do not definitively decrease morbidity and mortality in patients with HFpEF (Borlaug, 2020). However, there are weak signals of benefit for mineralocorticoid receptor antagonists (Borlaug, 2020). It is important to note that the Prospective Comparison of ARNI with ARB Global Outcomes in Heart Failure With Preserved Ejection Fraction (PARAGON-HF) trial, which is the latest and largest HFpEF outcomes trial, reported a strong sex difference in the efficacy of angiotensin receptor neprilysin inhibitor (ARNI) treatment, with greater benefits observed in women than in men (Solomon et al., 2019; McMurray et al., 2020). Sacubitril–valsartan, compared with valsartan, reduced the prevalence of cardiovascular death and total hospitalizations for HF by 27% in women with HFpEF, but with no effect in men (Solomon et al., 2019; McMurray et al., 2020).

The incidence of ischemic heart disease (IHD) is higher in men than in women throughout their lifespans, even though the sex difference decreases as age increases (Albrektsen et al., 2017). Despite the low prevalence of myocardial infarction in women compared to men, a recent large-scale cohort study showed that women have a higher risk of death and HF than men in the 5 years following an ST-segment-elevation myocardial infarction, even after accounting for differences in angiographic findings, revascularization, and other confounders (Ezekowitz et al., 2020). Women with IHD characteristically have higher prevalence of angina, burden of CVD risk factors, and prevalence of non-obstructive coronary artery disease on angiography than men with IHD (Garcia et al., 2016). Non-obstructive coronary artery disease, also known as microvascular angina, is a disease that predominantly affects postmenopausal women (Jespersen et al., 2012), where estrogen is reported to mediate coronary microvascular function by modulating nitric oxide (NO) in coronary endothelium (Lu et al., 2016; Vanhoutte et al., 2016). CHIP is associated with elevated levels of inflammatory cytokines and accelerated atherosclerosis in animal and human studies (Fuster et al., 2017; Jaiswal et al., 2017; Jaiswal and Libby, 2020). A recent study reported that premature menopause (i.e., menopause before the age of 40), and especially natural premature menopause, is independently associated with increased risk of CHIP (Honigberg et al., 2021). This suggests that CHIP is associated with incident coronary artery disease events in postmenopausal middle-aged women independent of conventional coronary artery disease risk factors.

Although the risk of atrial fibrillation (AF) is higher in men than in women (Ball et al., 2018), it is well documented that women with AF have higher risks of stroke, myocardial infarction and HF than men with AF (Regitz-Zagrosek et al., 2016). In the CHA_2DS_2-VASc scoring system used to evaluate the risk of stroke, a point is added for female sex, and patients with total points ≥ 2 who have another risk factor are recommended to receive oral anticoagulant therapy to prevent stroke (January et al., 2014; Kirchhof et al., 2016). Uncontrolled systolic hypertension is a stronger risk factor of incident AF in women than in men, associated with a twofold increased risk

of incident AF in women and a 30–60% increased risk in men (Sharashova et al., 2020).

Hormone Therapy in Cardiovascular Diseases

These sex differences in CVD prevalence may be attributed to estrogen function in cardiovascular organs, and this is supported by studies conducted over previous decades. In 1978, the Framingham study reported that women with surgical menopause have a 2.7-fold higher risk of CVD events than women of the same age without surgical menopause (Gordon et al., 1978). This finding led to the notion that exogenous estrogen could reduce the risk of CVD events in postmenopausal women. Several cohort studies consistently reported the cardioprotective effect of hormone therapy (HT) that lowers risk of CVD (Grodstein et al., 1997; Varas-Lorenzo et al., 2000; Taylor et al., 2020). In turn, major randomized controlled trials reported around the year 2000 showed neutral effects of HT (Hulley et al., 1998; Grady et al., 2002), and a randomized placebo-controlled studies conducted by the Women's Health Initiative (WHI) reported no benefits in CVD prevention but observed rather increased risks of stroke and deep vein thrombosis (Rossouw et al., 2002). These conflicting results may reflect differences in the time between menopause and the start of HT. Earlier cohort studies have included younger women who underwent HT in the early postmenopausal period, while the randomized studies included participants who received HT 10 years after menopause when responsiveness to estrogen in cardiovascular tissues may have diminished.

In fact, recent studies provided evidence supporting this 'timing hypothesis'. The WHI-Coronary Artery Calcium Study (CACS) analyzed the calcified plaque burden on coronary arteries in women close to the age of menopause (50–59 years) who received estrogen or placebo. The women who received estrogen were found to have a lower calcified plaque burden than the women who received placebo (Manson et al., 2007). The Danish Osteoporosis Prevention Study (DOPS) was conducted to estimate the effects of early initiated HT on CVD prevention (Schierbeck et al., 2012). In DOPS, healthy women ($n = 1{,}006$) with a mean age of 49.7 years were randomly divided into two groups: HT group ($n = 502$) and no-treatment group ($n = 504$). Women treated with HT for 10 years had a significantly reduced risk of HF, myocardial infarction and mortality, but they did not have a significant increase in the risk of venous thromboembolism, stroke or cancer (Schierbeck et al., 2012). In the Early versus Late Intervention Trial with Estradiol study (ELITE), participants who had early menopause (<6 years after menopause) and those who had late menopause ($\geq$ 10 years after menopause) were randomized to receive oral 17β-estradiol or a placebo (Hodis et al., 2016). The carotid intima-media thickness (CIMT) measured by ultrasound was the primary clinical outcome as an estimation of cardiovascular risk. 17β-estradiol-treated early menopausal subjects had slower progression of CIMT than placebo-treated subjects, but there was no estrogen effect in late menopausal participants (Hodis et al., 2016). Taken together, these clinical findings suggest that estrogen HT exhibits cardioprotective effects when initiated at an ideal timepoint after menopause, encouraging the researchers to further investigate the molecular and physiological functions of estrogen and estrogen receptor (ER)-mediated signaling in the cardiovascular system.

The effects of sex hormones other than estrogen on CVD have not necessarily been evaluated sufficiently. Progesterone, in combination with estrogen, is effective in inhibiting endometrial hyperplasia and cancer (Beresford et al., 1997). The risk of CVD was lower when progesterone was used in combination with estrogen than with estrogen alone (Grodstein and Stampfer, 1995), suggesting that progesterone may have cardioprotective effects. However, the effects of progesterone itself on the cardiovascular system have been little studied so far. It has also been reported that low serum testosterone levels are associated with an increase of the incidence of CVD in men (Khera et al., 2021), while exogenous testosterone therapy reportedly increases the risk of cardiovascular disease (Basaria et al., 2010; Vigen et al., 2013), so the cardiovascular actions of androgens need to be further studied as well.

MOLECULAR MECHANISMS OF ESTROGEN RECEPTOR SIGNALING IN CARDIOVASCULAR CELLS

There are two ERs: ERα and ERβ, both of which exhibit high homology (Mendelsohn and Karas, 1999). Ligand-bound ERs translocate from cytoplasm to nucleus and regulate gene expression as transcription factors (nuclear ER signaling). ERs alternatively function without nuclear translocation via enzymatic signaling pathways (non-nuclear ER signaling) (Mendelsohn and Karas, 2010; Ueda and Karas, 2013). Functional ERs are expressed in various cardiovascular cell types of humans and animals, including vascular endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and cardiomyocytes (Mendelsohn and Karas, 1999). Estrogen is also known to signal via a transmembrane G-protein-coupled receptor known as GPER. The characteristics and signaling targets of each ER are summarized in **Table 1**. Since GPER has been reviewed extensively in other papers (Haas et al., 2009; Prossnitz and Barton, 2011; Feldman and Limbird, 2017; Luo and Liu, 2020), we will focus on ERα and ERβ in this review.

In the nucleus, ligand-bound ERs function as transcription factors, interacting with estrogen response elements, and thereby regulate gene expression (Mendelsohn and Karas, 2005). Also, nuclear ER-estrogen complexes modulate the function of other transcription factor classes via protein–protein interactions. Hence, these complexes control gene expression without directly binding to DNA (Mendelsohn and Karas, 1999; McKenna and O'Malley, 2002). Recruitment of co-activators and displacement of co-repressors differ in each cell type, which determine cellular response to estrogen.

Cellular physiological responses to estrogen are elicited within minutes by the activation of membrane-associated ER, which has been termed "rapid" or "non-nuclear" ER signaling (Ueda and Karas, 2013). Non-nuclear ER signaling has been

TABLE 1 | Characteristics of ERs.

ERs	ERα		ERβ		GPER
Identification	1969		1996		1997
Category	Nuclear steroid hormone superfamily				G protein-coupled receptor superfamily
Location	Cytoplasm, nucleus	Membrane (caveolae)	Cytoplasm, nucleus	Membrane (caveolae)	Membrane
Targets	ERE, non-ERE	PI3K, ERK	ERE, non-ERE	ND	AC/PKA, EGFR (PI3K, ERK)
References	(Caulin-Glaser et al., 1997; Lantin-Hermoso et al., 1997; Mendelsohn and Karas, 1999, 2005, 2010; Chambliss et al., 2000, 2010; Simoncini et al., 2000; McKenna and O'Malley, 2002; Florian et al., 2004; Lu et al., 2004; Levin, 2005; Osborne and Schiff, 2005; Pedram et al., 2006; Ueda and Karas, 2013; Ueda et al., 2018)		(Mendelsohn and Karas, 1999, 2005; Chambliss et al., 2002; McKenna and O'Malley, 2002; Patten et al., 2004; Fliegner et al., 2010)		(Haas et al., 2009; Prossnitz and Barton, 2011; Feldman and Limbird, 2017; Luo and Liu, 2020)

ER, estrogen receptor; GPER, G protein estrogen receptor; ERE, estrogen response element; AC, adenylate cyclase; PKA, protein kinase A; EGFR, epidermal growth factor receptor; PI3K, phosphoinositide 3-kinase; ERK, extracellular signal-regulated kinase; ND, not determined.

identified in various cell types *in vitro*, including VSMCs, ECs, and cardiomyocytes (Osborne and Schiff, 2005; Ueda and Karas, 2013). The ERs located in small invaginations of the cell membrane known as caveolae signal the rapid actions via activating kinases or phosphatases to affect cell physiology (Levin, 2005; Pedram et al., 2006). Non-nuclear ER signaling in the cardiovascular system has been most studied in ECs, where rapid (within 15–30 min) activation of endothelial nitric oxide synthase (eNOS) by estrogen was observed (Caulin-Glaser et al., 1997; Lantin-Hermoso et al., 1997). ERs that reside in caveolae activate PI3K, Akt and ERK1/2 kinases, leading to activation of eNOS phosphorylation in ECs (Simoncini et al., 2000; Florian et al., 2004; Pedram et al., 2006). ERα binds to striatin, which is a scaffold protein colocalized with caveolin-1. The activation of PI3K requires that striatin acts as the scaffold protein of the ERα complex at the caveolae (Chambliss et al., 2000; Lu et al., 2004). Blocking ERα-striatin binding, either with a peptide that represents ERα amino acids 176–253 or with the ERα triple-point mutation (lysine 231, arginine 233 and arginine 234 to alanine: KRR), abolishes non-nuclear signaling without affecting nuclear signaling (Lu et al., 2004, 2016; Bernelot Moens et al., 2012; Ueda et al., 2018). Meanwhile, endogenous ERβ was also found in the EC membrane, specifically at the caveolae; however, its associated proteins have not been determined (Chambliss et al., 2002).

ESTROGEN ACTIONS IN ANIMAL MODELS OF CARDIOVASCULAR DISEASES

Ischemic Heart Diseases

In animal models of IHDs, such as myocardial infarction and ischemia–reperfusion, both of ERα and ERβ were reported to play a role in the cardioprotective effects of estrogen. After myocardial infarction, increased mortality and HF exacerbation were observed in global ERβ KO mice (Pelzer et al., 2005). Consistently, cell-type specific overexpression of ERβ in cardiomyocytes improved cardiac function and survival after myocardial infarction. In female mice overexpressing ERα, cardiac fibrosis after myocardial infarction was inhibited with increased angiogenesis (Mahmoodzadeh et al., 2014; Schuster et al., 2016). In an ischemia–reperfusion model, estrogen normalized coronary endothelial dysfunction in ovariectomized wild-type mice, while estrogen failed to reverse it in global ERα KO mice (Favre et al., 2010). ERα KO mice also demonstrated markedly impaired cardiac contractility, increased cardiomyocyte death and mitochondrial damage after ischemia–reperfusion (Zhai et al., 2000; Wang et al., 2006). In contrast, in an *ex vivo* model of global ischemia–reperfusion, the hearts of female ERβ KO mice showed poor functional recovery compared to those of wild-type mice, but no significant difference was observed between ERα KO and wild-type mice (Gabel et al., 2005). Mechanistically, estrogen attenuates reperfusion injuries after ischemia mainly via activation of PI3K-Akt, increased expression of the anti-apoptotic protein BCL-2 and reduced expression of proapoptotic caspase proteins (Patten et al., 2004). In female ERβ KO mice, estrogen treatment failed to induce recovery from ischemic injury or activation of PI3K-Akt signaling in the hearts (Patten et al., 2004; Fliegner et al., 2010). Taken together, ERβ seems to play important roles in cardioprotection against ischemia–reperfusion injury, while the role of ERα varies depending on methodological conditions.

Cardiac Hypertrophy and Failure

Pathological cardiac hypertrophy develops in response to various pathological stresses, including genetic, mechanical and neurohormonal stress. Excessive and prolonged stress leads hypertrophy to failure. Sex difference is known as a modifier of cardiomyopathy in humans (van Berlo et al., 2013), as well as in genetically modified mouse models of hypertrophic cardiomyopathy, including a missense mutation (R403Q) in the α-myosin heavy chain and a missense mutation (R92Q) in cardiac troponin T (Maass et al., 2004; McKee et al., 2013; Chen et al., 2015). In both transgenic mice, male mice showed an overt phenotype of cardiac hypertrophy and failure compared

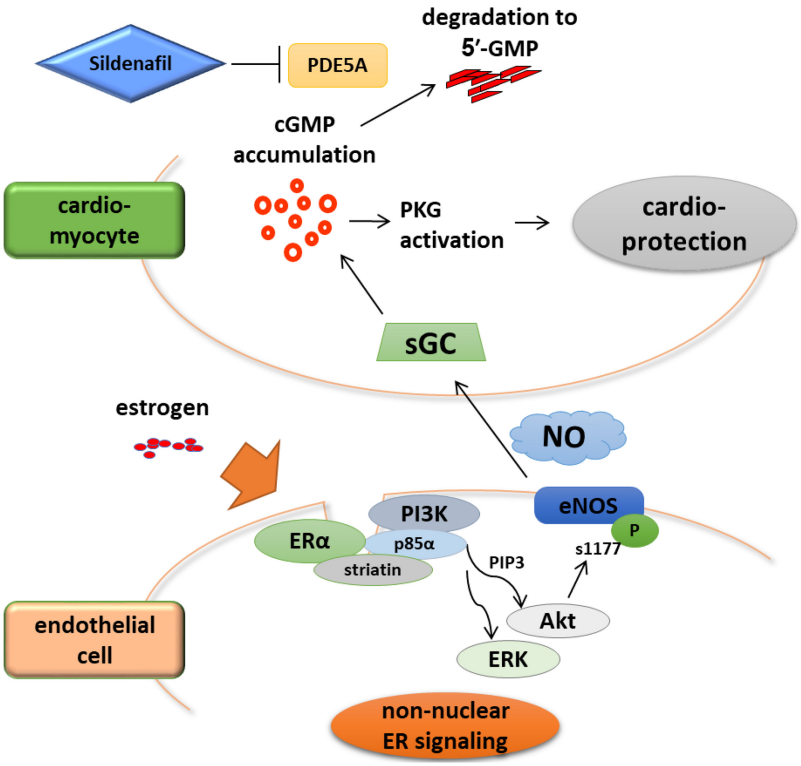

FIGURE 1 | Rapid non-nuclear ERα signaling is indispensable for estrogen to provide NO that activates sGC. ERα non-nuclear signaling requires the interaction between ERα and striatin, a scaffold protein residing at caveolae. A transgenic mouse line in which ERα non-nuclear signaling was selectively disrupted showed that ERα non-nuclear signaling was indispensable to the therapeutic efficacy of cGMP-PDE5 inhibition in heart failure but not to that of sGC stimulation. These data imply the advantage of sGC stimulation over PDE5 inhibition as a potential therapeutic strategy in treating heart failure in post-menopausal women, highlighting the need for female-specific therapeutic strategies.

with female mice (Olsson et al., 2001; Maass et al., 2004; McKee et al., 2013). Importantly, ovariectomized female mutant mice had worse phenotypes with greater impairment of contractile function and myocardial energy metabolism, while estrogen supplementation restored these parameters (Chen et al., 2015). These findings suggest protective effects of estrogen against cardiac hypertrophy and failure.

Results of studies that used global ERα or ERβ KO mice subjected to chronic angiotensin II treatment or pressure overload have suggested the role of ERβ in the protective property of estrogen against cardiac hypertrophy and failure. Mechanistically, the link between estrogen and the cGMP-PKG signaling pathway may be a key that deserves further investigation (Kim and Levin, 2006). Upregulation of cGMP signaling in myocardium has emerged as a novel therapeutic strategy for heart failure, evidenced by recent clinical studies. The Vericiguat Global Study in Subjects with Heart Failure with Reduced Ejection Fraction (VICTORIA) study showed cardiovascular protection by the soluble guanylate cyclase (sGC) stimulator vericiguat (Armstrong et al., 2020). Neprilysin inhibition by ARNI that provides cardiovascular benefits also stimulates cGMP signaling via augmentation of the natriuretic peptides (McMurray et al., 2014). Considering that myocardial cGMP-PKG signaling pathway is deactivated in human HFpEF and that HFpEF is associated with female sex independent of obesity and diabetes (Lee et al., 2009; Dunlay et al., 2017), it is reasonable to assume that estrogen decline and subsequent cGMP deactivation may contribute to the pathophysiology of HFpEF. In fact, estrogen signaling is crucial for a PDE5 inhibitor sildenafil-induced activation of cGMP-PKG in cardiac myocytes to ameliorate HF in female mice (Fisher et al., 2005; Sasaki et al., 2014). Additionally, using a novel knock-in mice, whose ERα are replaced with the ERα harboring triple-point KRR mutation, we recently reported that rapid non-nuclear ERα signaling is indispensable for estrogen to provide NO that activates sGC (**Figure 1**; Fukuma et al., 2020). These results suggest a potential link between estrogen and cGMP signaling. A recent study provided a great progress in the experimental research of HFpEF, where mice treated with a combination of high-fat diet and inhibition of NOS signaling by L-NAME recapitulates the systemic and cardiovascular features of human HFpEF (Schiattarella et al., 2019). In contrast to observations in humans, however, female mice in the HFpEF model developed a significantly attenuated cardiac phenotype compared with their male counterparts, and this protection in female mice was preserved even by ovariectomy (Tong et al., 2019). Given that ARNI use for HFpEF patients reduced the risk of HF only in women (Solomon et al., 2019; McMurray et al., 2020),

extended studies may clarify the molecular mechanisms by which cardiovascular benefits provided by the natriuretic peptide augmentation and its downstream cGMP signaling show the sex difference in HFpEF.

Injury Response in the Vasculature and Atherosclerosis

Vascular damage provokes regional vascular inflammation and prolonged inflammation leads to pathological vascular remodeling that manifests as neointimal hyperplasia. Estrogen was found to inhibit the intimal thickening in a mouse carotid artery injury model through inhibiting the proliferation of VSMCs and promoting re-endothelialization (Iafrati et al., 1997; Hayashi et al., 2000; Brouchet et al., 2001; Chambliss et al., 2010). In ERα KO mice, estrogen treatment failed to protect vasculature against the vascular injury (Brouchet et al., 2001; Pare et al., 2002), while in ERβ KO mice, it is still protective (Karas et al., 1999; Brouchet et al., 2001), suggesting that ERα is responsible for the estrogen protection on vasculature. The importance of the non-nuclear ER signaling pathway in estrogen-induced vascular protection has been evaluated in gain- and loss-of-function studies. Estrogen dendrimer conjugates (EDC), which was found to specifically bind to membrane ERs but not those in cytoplasm and selectively activates non-nuclear ER signaling, promoted re-endothelialization in injured carotid arteries in an ERα-dependent manner (Chambliss et al., 2010). Notably, endometrial carcinoma cell growth was activated by estrogen, but not EDC, suggesting that selective activation of the non-nuclear ER signaling does not promote cancer growth (Chambliss et al., 2010). In turn, estrogen's vascular protective effect was not observed in disrupting peptide mice (DPM), in which ERα-striatin binding was disrupted due to overexpression of a peptide that represents ERα amino acids 176–253 (Bernelot Moens et al., 2012), suggesting that non-nuclear signaling plays a substantial role in the protection by estrogen against vascular injury. Meanwhile, ligand-bound ERα mediates the transcription of target genes through the activation function 2 (AF2) domain, which is located on the C-terminal. Knock-in mice without a functional AF2 domain showed impaired estrogen protection against atherosclerosis (Billon-Galés et al., 2011). Conversely, the estrogen effects on re-endothelialization after vascular injury was preserved in these mice (Billon-Galés et al., 2011). Another study using a knock-in mouse model harboring a point mutation of the arginine 264 of ERα (R264A-ERα), in which non-nuclear ERα signaling is selectively abrogated, consistently showed that endothelial healing is mediated by non-nuclear ERα signaling, and in turn, atheroma protection is mediated by nuclear ERα action (Adlanmerini et al., 2020). Additionally, increased atherosclerotic lesion area was displayed in LDL receptor-KO mice transplanted with ERα KO mice bone marrow, suggesting a substantial role of ERα signaling in bone marrow cells for atheroprotection (Ribas et al., 2011).

CONCLUSION

Estrogen directly affects cardiovascular tissues and may have considerable influence on the sex differences observed in the epidemiology and outcomes of CVDs. Recent clinical studies have highlighted the diverse cardiovascular effects of estrogen, and research into the mechanisms of action of the sex hormone will be increasingly important in the future.

AUTHOR CONTRIBUTIONS

KU and ET wrote the manuscript. NF, YA, GN, HT, MT, AO, MH, and P-YL critically revised the manuscript and contributed to design the figure. All authors contributed to the article and approved the submitted version.

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Association Between Depression and Risk of Incident Cardiovascular Diseases and Its Sex and Age Modifications: A Prospective Cohort Study in Southwest China

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To examine possible associations between depression and cardiovascular disease (CVD) incidence and whether demographic factors modified those associations in the Chinese population. This prospective cohort study comprised 7,735 adults aged 18 years or older in Guizhou, China from 2010 to 2020. The Patient Health Questionnaire-9 (PHQ-9) was used to measure the prevalence of depression. Cox proportional hazard models were used to estimated hazard ratios (HRs) and 95% confidence intervals (95%CIs) of depression and incident CVD. We identified 215 CVD cases (including 28 acute myocardial infarction (AMI) and 197 stroke cases) during an average follow-up of 7.07 years. In the multivariable-adjusted model, baseline PHQ-9 score was associated with incident CVD, AMI, and stroke. The HR per 1-SD increase for PHQ-9 score was 1.14 (95%CI: 1.03, 1.26) for CVD, 1.26 (95%CI: 1.01, 1.57) for AMI, and 1.12 (95%CI: 1.01, 1.25) for stroke. Compared with participants without depression, those with any mild or more advanced depression had a higher risk of incident CVD (HR: 1.69, 95%CI: 1.08, 2.64) and AMI (HR: 3.36, 95%CI: 1.17, 10.56). Associations between depression with CVD and stroke were suggested to be even stronger among women and participants aged <65 years (*P* for interaction <0.05). The effect of depression on stroke tended to be preserved in non-smokers. Depression was associated with a higher risk of incident CVD, AMI, and stroke in adults of Southwest, China, particularly in women, participants aged <65 years, and non-smokers. These findings highlighted the importance and urgency of depression healthcare.

Keywords: the Patient Health Questionnaire-9 (PHQ-9), depression, cardiovascular disease, effect modification, cohort study

INTRODUCTION

Depression is a leading cause of disability, with more than an estimated 264 million people affected worldwide (1). Previous studies have reported that depression is consistently associated with a higher risk of adverse cardiovascular disease (CVD). In a meta-analysis of 28 prospective cohort studies, Pan et al. (2) reported a pooled adjusted hazard ratio (HR) of 1.45 for incident stroke

associated with baseline depression. Another systematic review reported that people with major depression have a 56% higher risk of developing ischemic heart disease (IHD), with depression accounting for 2.95% of the disability-adjusted life years associated with IHD (3). However, the causal relationship between depression and CVD is still questionable, while the previous positive association was largely based on cross-sectional studies, cohort studies with short follow-up durations, or with inadequate adjustment of potential confounding factors. Also, the results had been inconsistent between different sociodemographic strata, such as men and women. For example, a meta-analysis found similar associations between depression and stroke in both men and women (4), while a study in Sweden suggested that the effect of depression on stroke was higher in men compared with women (5). The previous study has found a stronger association between depression and stroke in participants aged <65 years but not in participants ≥65 years (6). Therefore, more prospective cohort studies are still needed to examine whether the association between depression and CVD differs over sociodemographic factors.

To our knowledge, very few prospective cohort studies have been conducted on this issue among Chinese (7–9), in which three cohort studies covered middle-aged and older Chinese adults (7–9), and studies including younger adults in China were still not reported so far. In this study, we used data from a prospective cohort study in Southwest China to investigate whether depression was associated with CVD in adults and test whether those associations were modified by sociodemographic factors.

MATERIALS AND METHODS

Study Design and Population

The Guizhou Population Health Cohort Study (GPHCS) was a prospective community-based cohort in Guizhou province located in Southwest China. A total of 9,280 residents was enrolled from 48 townships of 12 districts (or counties) in this cohort from 2010 to 2012 using a multistage proportional stratified cluster sampling method. The inclusion criteria included residents aged 18 years or older, who had no plan to move out and completed survey questionnaire and blood sampling. All participants were followed up for major chronic diseases and vital status through a repeated investigation from 2016 to 2020 with the loss to follow-up rate of 12.04%. A total of 428 participants were further excluded for this analysis, including 44 with a history of CVD, 214 without reliable information on CVD status at follow-up, and 170 without sufficient information on depression at baseline. This study was approved by the Institutional Review Board of Guizhou Center for Disease Control and Prevention (No. S2017-02). All participants signed the written informed consent.

Assessment of Depressive Symptoms

The Patient Health Questionnaire-9 (PHQ-9) with a 9-question depression scale, was used to screen for the presence and severity of depressive symptoms according to the Diagnostic and Statistical Manual of Mental Disorders-IV criteria (DSM-IV) (10). Subjects were asked to respond to each symptom by rating the best statement applied over the past 2 weeks, using a score from zero to three (ranging from "not at all" = zero, "several days" = one, "more than half the days" = two, or "nearly every days" = three). Given a range of total scores between 0 and 27, the higher score indicated the greater severity of depressive symptoms. They were divided into three categories according to the PHQ-9 scores (0, no depression; 1 to 4, minimal depressive symptoms; and ≥5, mild or more advanced symptoms as depression) (11). The Chinese version of the PHQ-9 has demonstrated high reliability and validity (12).

Ascertainment of Incident CVD Events

The study outcome was self-reported incident CVD events. Incident CVD events were assessed by the following standardized questions: "Have you been diagnosed with cerebral hemorrhage by a doctor?", "Have you been diagnosed with subarachnoid hemorrhage by a doctor?," "Have you been diagnosed with cerebral infarction by a doctor?", or "Have you been diagnosed with acute myocardial infarction (AMI) by a doctor?" Participants who reported cerebral hemorrhage, subarachnoid hemorrhage, or cerebral infarction during the follow-up period were defined as having an incident stroke, and those who reported the above symptoms or AMI were defined as having incident CVD. All deaths were confirmed by the Death Registration Information System and Basic Public Health Service System, and deaths from AMI or stroke were considered as incident CVD cases.

Covariates

Information on the covariates was collected by trained health workers using a structured questionnaire via a face-to-face interview, including sociodemographic characteristics (age, sex, ethnicity, education, marriage status, and occupation), lifestyle (smoking status, alcohol use, and physical activity), history of chronic diseases (type 2 diabetes (T2DM), hypertension, and dyslipidemia), and use of medications for T2DM, hypertension, and dyslipidemia. Height, body weight, and blood pressure were measured by trained health workers. Current smoker was defined as smoking at least one cigarette or other tobacco product a day for 12 months or more. Alcohol use was defined as drinking at least one time a week for 12 months or more. Physical activity was defined as having moderate or vigorous physical activity at least 10 min every time for one or more times per week. Body mass index (BMI) was calculated as body weight in kilograms divided by square height in meters (kg/m^2). Venous blood samples were obtained from participants after at least 8 h overnight fast to measure fasting plasma glucose (FPG), total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL), and low-density lipoprotein cholesterol (LDL-C). A 2-h oral glucose tolerance test (OGTT) with 75 g of glucose was carried out for participants. T2DM was defined if participants met either of the following criteria: (1) self-reported doctor diagnosis of diabetes or use of anti-diabetic medications; (2) FPG ≥7.0mmol/L; (3) OGTT ≥11.1 mmol/L; (4) HbA1c ≥6.5% (13). Hypertension was defined as who met either of the following

criteria: (1) systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg; and/or (2) self-reported doctor diagnosis of hypertension or use of hypertension medications (14). Dyslipidemia was defined as who met either of the following criteria: (1) self-reported doctor diagnosis of dyslipidemia or use of lipid regulating drugs; (2) high TC: TC ≥6.22 mmol/L; (3) high TG: TG ≥2.26 mmol/L; (4) low HDL-C: HDL-C <1.04 mmol/L; 5) high LDL-C: LDL-C ≥4.14 mmol/L (15).

Statistical Analysis

Data were described as means and SDs for continuous variables, and as frequencies and percentages for categorical variables. Baseline characteristics are summarized according to depression status and compared using the analysis of variance or the Chi-square test. The person-years (PYs) of follow-up were calculated for each participant from the date of enrolling the cohort to the date of the CVD diagnosis, death, or the date of follow-up, whichever came first. The associations of depression with CVD, AMI, and stroke were estimated using Cox proportional hazards regression models. Two models were estimated: (1) Model 1: age (<30, 30–39, 40–49, 50–59, 60–69, ≥70) and sex were adjusted; (2) Model 2: the variables in model 1 plus ethnicity (Han Chinese or non-Han Chinese), education (<9 or ≥9 years), marriage status (married or other), occupation (framer or other), smoking status (current smoker or non-smoker), alcohol use (yes or no), physical activity (yes or no), BMI, history of T2DM (yes or no), history of hypertension (yes or no), and history of dyslipidemia (yes or no) were adjusted. To assess the robustness of the results, the following sensitivity analyses were performed: (1) We repeated Model 2 after excluding participants who were followed up less than 2 years, and (2) Considering the competing risk of death, a competing risk model was also fitted. The potential effect modifications by age (<65 or ≥65 years old), sex, smoking status, alcohol use were estimated by (1) including multiplicative interaction terms in the multivariable Cox models; (2) fitting stratified models. We used the Schoenfeld residuals to test the assumption of hazard proportionality in Cox regression models and found no evidence of non-proportionality ($P \geq 0.05$). Two-sides $P < 0.05$ was considered statistically significant. All analyses were performed in R software (Version 4.0.3; R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Study Participants

Of 7,735 eligible subjects for the current analysis (**Figure 1**), 47.7% were men, with an average age of 44.37 ± 15.07 years old. A total of 500 (6.46%) participants was presented with depression (PHQ-9 score ≥ 5), with an average score of 7.22 ± 2.88, and near one-fifth (19.0%) had minimal depression with the PHQ-9 score between 1 to 4. Compared with participants without depression (PHQ-9 score = 0), depressive ones were older, women, ethnic minority, farmers, or having lower education levels (**Table 1**). They also had a lower proportion of current smokers and a higher prevalence of hypertension or dyslipidemia.

Associations of Depression With Incident CVD, AMI, and Stroke

During the mean of 7.07 follow-up years, a total of 215 new CVD cases were identified with the crude incident density of 3.93 per 1,000 PYs, with 28 (0.51 per 1,000 PYs) new AMI cases and 197 (3.60 per 1,000 PYs) stroke cases (**Table 2**). The crude incident density was highest in the depression group (6.55 per 1,000 PYs), followed by minimal, and no depression groups. The age- and sex-adjusted Cox model showed that the PHQ-9 score was associated with an increased risk of incident CVD, AMI, and stroke. In the fully adjusted models, the adjusted HRs were 1.14 (95%CI: 1.03, 1.26) for CVD, 1.26 (95%CI: 1.01, 1.57) for AMI, and 1.12 (95%CI: 1.01, 1.25) for stroke with per SD increase of PHQ-9 score. Compared with no depression participants, those with minimal depression experienced a statistically increased risk of incident AMI, and those with depression had a higher risk of incident CVD and AMI.

In the sensitivity analysis (**Table 3**), the corresponding effect estimates of baseline depression status on the incident CVD and AMI did not change substantially after excluding participants who were diagnosed with CVD or AMI within 2 years after entering the cohort. However, the association between depression and incident stroke was attenuated, with the adjusted HR of 1.11 (95%CI: 0.99, 1.26) for per SD increase of PHQ-9 score. When the competing risk model was used to estimate the associations between depression with incident CVD, AMI, and stroke, the effects were similar to those in the main analysis.

Subgroup Analysis and Effect Modification

We also explored the potential effect modification of baseline age, sex, smoking status, and alcohol use on the associations of depression with incident CVD, AMI, and stroke, and the results of the subgroup analyses were presented in **Figure 2**. The effects of PHQ-9 score on CVD and stroke were higher in women or participants aged <65 years than men or those aged ≥65 (P for the interaction of CVD and stroke <0.05). The associations between PHQ-9 score with stroke were stronger in non-smokers (P for the interaction <0.05). However, alcohol use modification was not significant.

DISCUSSION

This study examined the associations between depression and incident CVD, AMI, and stroke in a prospective cohort study of 7,735 adults in Southwest China with an average of 7 years follow-up. At baseline, 6.5% of the participants experienced a mild or more advanced depression. PHQ-9 score was associated with risk of incident CVD, AMI, and stroke, while depression was associated with 1.69-fold and 3.36-fold risks of CVD and AMI, respectively. Furthermore, we found that the associations were only significant in women, participants aged <65 years, non-smokers, and non-alcohol users.

Previous studies have suggested that depression is associated with an increased risk of CVD (2, 3, 7, 8, 16, 17). In the Jackson Heart Study among 3,309 participants followed-up for 10 years, O'Brien et al. found that major depressive symptoms, defined as

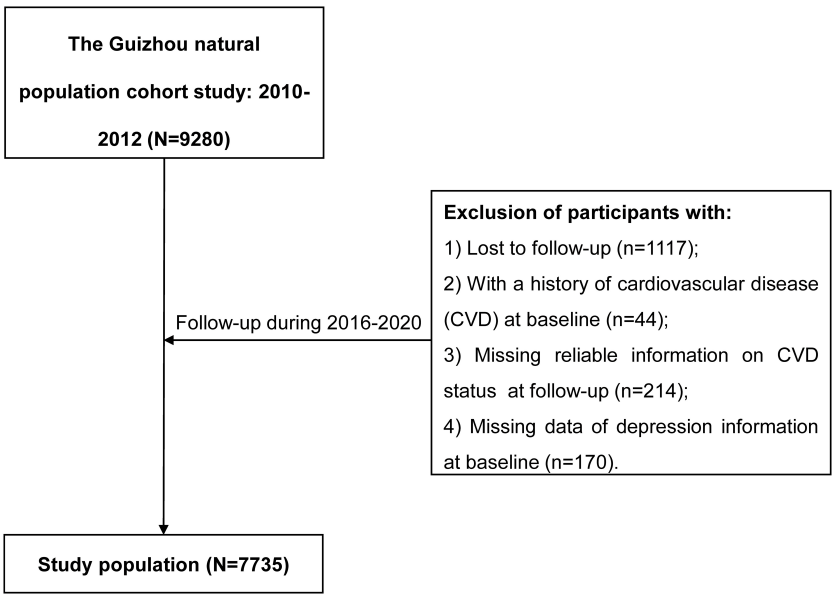

FIGURE 1 | The flow chart.

TABLE 1 | General characteristics of the study population by the depression status at baseline in Southwest China.

	Total (*N* = 7,735)	**Depression status**			***P-value***
		No (0)	**Minimal (1–4)**	**Mild or more advanced (≥5)**	
N	7,735	5,766	1,469	500	
PHQ-9 score	0.87 ± 2.05	0	2.11 ± 1.05	7.22 ± 2.88	<0.001
Age at baseline, years	44.37 ± 15.07	43.52 ± 14.95	46.80 ± 15.28	47.12 ± 14.78	<0.001
<30	1,517 (19.6)	1,213 (21.0)	236 (16.1)	68 (13.6)	<0.001
30.0–39.9	1,655 (21.4)	1,293 (22.4)	271 (18.4)	91 (18.2)	
40.0–49.9	1,942 (25.1)	1,433 (24.9)	369 (25.1)	140 (28.0)	
50.0–59.9	1,331 (17.2)	945 (16.4)	289 (19.7)	97 (19.4)	
60.0–69.9	824 (10.7)	569 (9.9)	190 (12.9)	65 (13.0)	
≥70.0	466 (6.0)	313 (5.4)	114 (7.8)	39 (7.8)	
Men, %	3,692 (47.7)	2,851 (49.4)	645 (43.9)	196 (39.2)	<0.001
Ethnic minority, %	3,197 (41.3)	2,471 (42.9)	541 (36.8)	185 (37.0)	<0.001
Education ≥9 years, %	3,328 (43.0)	2,618 (45.4)	544 (37.0)	166 (33.2)	<0.001
Married, %	6,251 (80.8)	4,631 (80.3)	1,224 (83.3)	396 (79.2)	0.021
Farmer, %	4,411 (57.0)	3,379 (58.6)	787 (53.6)	245 (49.0)	<0.001
Current smoker, %	1,972 (25.5)	1,502 (26.0)	364 (24.8)	106 (21.2)	0.045
Alcohol use, %*	1,525 (19.7)	1,146 (19.9)	299 (20.4)	80 (16.0)	0.089
Physical activity, %	67,41 (87.1)	5,023 (87.1)	1,311 (89.2)	407 (81.4)	<0.001
BMI, kg/m^2	22.90 ± 3.36	22.90 ± 3.35	23.01 ± 3.46	22.55 ± 3.12	0.030
History of T2DM, % *	657 (8.5)	486 (8.4)	130 (8.8)	41 (8.2)	0.202
History of hypertension, %	2,014 (26.0)	1,451 (25.2)	426 (29.0)	137 (27.4)	0.032
History of dyslipidemia, %	4,436 (57.3)	3,258 (56.5)	874 (59.5)	304 (60.8)	0.009

**missing value.*

PHQ-9, Patient Health Questionnaire-9; BMI, body mass index; T2DM, type 2 diabetes mellitus.

TABLE 2 | The incident risk of CVD, AMI, and Stroke associated with baseline depression status.

	Cases, *n*	Incident density/1000 PYs	Hazard Ratio (95% Confidence Interval)	
			Mode 1	Mode 2
CVD				
PHQ-9 score (per SD increase)	215	3.93	1.14 (1.03, 1.26) *	1.14 (1.03, 1.26) *
No (0)	146	3.57	1.00	1.00
Minimal (1–4)	46	4.47	1.11 (0.79, 1.55)	1.10 (0.78, 1.53)
Mild or more advanced (≥5)	23	6.55	1.64 (1.05, 2.55) *	1.69 (1.08, 2.64) *
AMI				
PHQ-9 score (per SD increase)	28	0.51	1.26 (1.02, 1.55) *	1.26 (1.01, 1.57) *
No (0)	13	0.32	1.00	1.00
Minimal (1–4)	11	1.06	3.05 (1.36, 6.84) **	3.11 (1.37, 7.07) **
Mild or more advanced (≥5)	4	1.13	3.36 (1.09, 10.42) *	3.36 (1.17, 10.56) *
Stroke				
PHQ-9 score (per SD increase)	197	3.60	1.12 (1.00, 1.25) *	1.12 (1.01, 1.25) *
No (0)	137	3.35	1.00	1.00
Minimal (1–4)	40	3.89	1.02 (0.72, 1.46)	0.99 (0.70, 1.42)
Mild or more advanced (≥5)	20	5.69	1.51 (0.94, 2.42)	1.55 (0.96, 2.49)

Model 1: adjusted for age (<30, 30–39, 40–49, 50–59, 60–69, ≥70), sex.
Model 2: model 1 plus ethnicity, education, marriage, occupation, smoking status, alcohol use, physical activity, history of T2DM, history of hypertension, history of dyslipidemia, and body mass index.
***P < 0.01, *P < 0.05.*
PY, person years; CVD, cardiovascular disease; PHQ-9, Patient Health Questionnaire-9; SD, standard deviation; AMI, acute myocardial infarction.

TABLE 3 | Sensitivity analysis.

	Hazard Ratio (95% Confidence Interval)		
	CVD	AMI	Stroke
Excluding participants who were diagnosed within 2 years			
PHQ-9 score (per SD increase)	1.13 (1.02, 1.26) *	1.26 (1.01, 1.57) *	1.11 (0.99, 1.26)
No (0)	1.00	1.00	1.00
Minimal (1–4)	1.21 (0.86, 1.72)	3.51 (1.53, 8.07) **	1.12 (0.77, 1.63)
Mild or more advanced (≥5)	1.63 (1.00, 2.66) *	3.62 (1.14, 11.47) *	1.50 (0.88, 2.54)
Competing risk model			
PHQ-9 score (per SD increase)	1.13 (1.03, 1.24) **	1.25 (1.04, 1.51) *	1.12 (1.01, 1.24) *
No (0)	1.00	1.00	1.00
Minimal (1–4)	1.09 (0.78, 1.52)	3.26 (1.42, 7.47) **	1.00 (0.70, 1.42)
Mild or more advanced (≥5)	1.64 (1.06, 2.53) *	3.26 (1.00, 10.67)	1.53 (0.96, 2.44)

Adjusted for age (<30, 30–39, 40–49, 50–59, 60–69, ≥70), sex, ethnicity, education, marriage, occupation, smoking status, alcohol use, physical activity, history of T2DM, history of hypertension, history of dyslipidemia, and body mass index.
***:P < 0.01, *P < 0.05.*
CVD, cardiovascular disease; AMI, acute myocardial infarction; PHQ-9, Patient Health Questionnaire-9; SD, standard deviation.

a score of 21 or higher on the 20-item Center for Epidemiological Studies Depression Scale (CES-D), were associated with a 2-fold greater hazard of stroke, while a per-SD increase in CES-D score was associated with a 1.3-fold (16). In another cohort of the China Health and Retirement Longitudinal study among 12,417 middle-aged and older adults, Li et al. (8) reported that participants with elevated depressive symptoms had a 39% (95%CI: 22, 58%) higher risk of incident CVD, a 36% (95%CI: 18, 57%) higher risk of heart disease, and a 45% (95%CI: 6, 99%) higher risk of stroke during the 4 years of follow-up. A recent meta-analysis of 21 studies involving 47, 625 participants found that participants with depressive symptoms had a 1.36-fold higher risk of stroke, but not of MI (HR: 1.08, 95%CI: 0.91, 1.29) (18). As expected, the depressive symptoms were associated with a higher risk of CVD and AMI in this study, while the association was not statistically significant in stroke. Apart from differences in methods of depression symptoms assessment, different age distribution, the residual confounding effects, divergent medical, behavioral, or social responses to the depressive disorder may partly explain the different findings overstudies. Another potential explanation for such differences may be the limited number of new cases in this study. Thus,

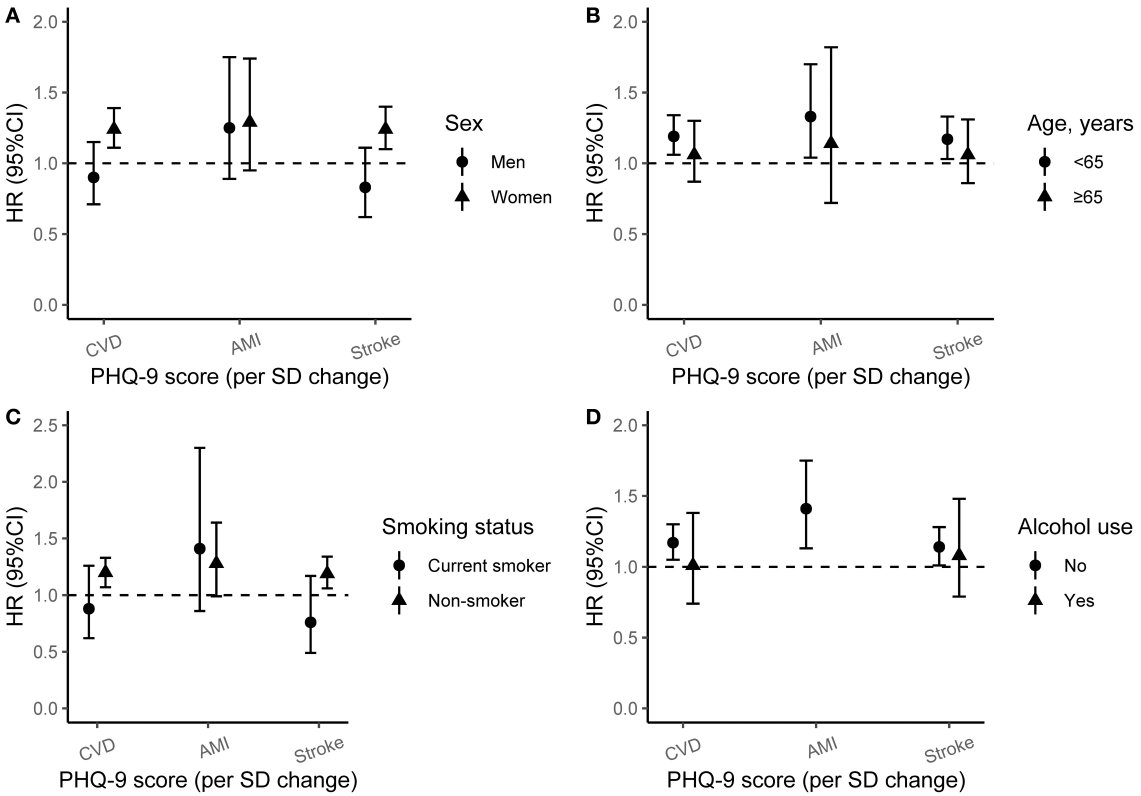

FIGURE 2 | The incident risk of CVD, AMI, and Stroke associated with baseline PHQ-9 score by sex, age, smoking status, and alcohol use. Adjusted for age (<30, 30–39, 40–49, 50–59, 60–69, ≥70), sex, ethnicity, education, marriage, occupation, smoking status, alcohol use, physical activity, history of T2DM, history of hypertension, history of dyslipidemia, and body mass index. HR, hazard ratio; 95%CI, 95% confidence interval; PHQ-9, Patient Health Questionnaire-9; SD, standard deviation; CVD, cardiovascular disease; AMI, acute myocardial infarction. **(A)** Subgroup analysis by sex; **(B)** subgroup analysis by age group; **(C)** subgroup analysis by smoking status; **(D)** subgroup analysis by alcohol use.

future work with a longer follow-up period is needed to confirm the association between depression and risk of CVD including AMI and stroke.

There are several potential mechanisms for the association of depression with excess risk of incident CVD. Biologically, depression is associated with hypothalamic-pituitary-adrenal axis hyperactivity (19), platelet activation (20), and immunological/inflammation effects (21), all of which might be linked to the increased CVD risk. Secondly, the depressive population often has unhealthy lifestyles, including smoking (22), alcohol abuse (23), low physical activity (24), and obesity (25), which could affect the occurrence of CVD. In addition, depression is associated with other comorbidities (26), like hypertension and diabetes, both are risk factors related to incident CVD. Nevertheless, after adjusting for baseline smoking status, alcohol use, physical activity, BMI, history of hypertension, and diabetes, the association between depression and incident CVD remained stable in this study, indicating that the effect of depression was independent of those risk factors mentioned above.

Sex modified associations between depression, and incident CVD and stroke in this study, which was inconsistent with several previous studies (4, 5, 8, 27). Those associations were more evident in women in this study. Hamano et al. evaluated sex differences in the association between depression and stroke and found that the effect of depression on stroke was higher in men compared with women (8). A meta-analysis of 17 prospective studies reported that the associations were similar between men and women (4). Even so, our findings provided new evidence that there may be a sex difference in the association of depression with incident CVD and stroke in the Chinese population. Although the exact mechanisms are still unclear, there are several potential biological and psychosocial explanations. First, the prevalence of depression is higher in women than men in this study. Compared with women, men may be less inclined to report a depressive disorder or seek help until the depression is severe (28, 29). Second, depression increases the plasma concentration of 5-hydroxytryptamine (5-HT), which is of particular relevance to women (20). 5-HT may affect platelet function and lead to platelet aggregation as well as coronary vasoconstriction (30). In addition, lifestyle differences may contribute to the stronger association in women. Previous studies have reported that there are several different risk factors associated with CVD in men and women, although the underlying biological mechanisms are still unclear (31, 32). The risk-elevating effect of depression on stroke tended to be

preserved in a subgroup of non-smokers, which might be due to most smokers being men.

Previous cohort studies in the Chinese population were among middle-aged and older adults (7, 8), while this study included participants aged 18 years or above. In the stratified analysis by age, we found that the effects of depression on incident CVD and stroke were higher in participants aged <65 years, which was consistent with the previous study (6). The Framingham Study found that depression increased the risk of stroke in those aged <65 years but not in those aged ≥65 years. More prospective studies with a large sample size are calling to confirm whether age modifies the association between depression and incident CVD in the future.

The strengths of this study included the well-characterized prospective design and the longer follow-up period with a relatively low loss to follow-up rate. To our knowledge, this is the first report on the association between depression and incident CVD in different demographic groups in Southwest China. This study also had notable limitations. Firstly, we only measured baseline depression status using PHQ-9 and did not measure during the follow-up. Also, we did not have clinical diagnoses information of depression, which might lead to a misclassification of the depression status. Secondly, those with depressive disorders may be less likely to participate owing to their loss of interest in most things. Thirdly, the outcome was self-reported and the timing of onset may be inaccurate, and the association might be underestimated. In addition, even though current analyses adjusted for major potential confounding factors, residual confounding resulting from dietary factors was still possible.

In conclusion, depression significantly increased the risk of incident CVD including AMI and stroke, especially in women, participants aged <65 years, and non-smokers. Further prospective studies with clinically diagnosed depression and repeated measures of depression in Chinses population are required to examine the potential underlying mechanisms. Our findings highlight the need for focused attention on increasing awareness and improving the healthcare of depression, and also suggest that primary care physicians should pay more attention to CVD prevention if females, the elder, or non-smokers become depressed.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, upon a reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Guizhou Center for Disease Control and Prevention. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LY and YC: conceptualization, methodology, formal analysis, validation, writing—original draft, and visualization. NW and KX: conceptualization, methodology, data curation, writing—review, and editing. CW: methodology, data curation, writing—review, and editing. TL: conceptualization, methodology, supervision, funding acquisition, writing—review, and editing. CF: conceptualization, methodology, supervision, resources, writing—review, and editing. All authors contributed to the article and approved the submitted version.

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