DIABETIC CARDIOMYOPATHY

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Sex-specific effects of daily tadalafil on diabetic heart kinetics in RECOGITO, a randomized, double-blind, placebo-controlled trial

Riccardo Pofi[1]†, Elisa Giannetta[1]†, Tiziana Feola[1,2], Nicola Galea[1], Federica Barbagallo[1], Federica Campolo[1], Roberto Badagliacca[3], Biagio Barbano[4], Federica Ciolina[5], Giuseppe Defeudis[6], Tiziana Filardi[1], Franz Sesti[1], Marianna Minnetti[1], Carmine D. Vizza[3], Patrizio Pasqualetti[7], Pierluigi Caboni[8], Iacopo Carbone[5], Marco Francone[5], Carlo Catalano[5], Paolo Pozzilli[6], Andrea Lenzi[1], Mary Anna Venneri[1], Daniele Gianfrilli[1]*‡, Andrea M. Isidori[1]*‡

Cyclic GMP–phosphodiesterase type 5 (PDE5) inhibition has been shown to counteract maladaptive cardiac changes triggered by diabetes in some but not all studies. We performed a single-center, 20-week, double-blind, randomized, placebo-controlled trial (NCT01803828) to assess sex differences in cardiac remodeling after PDE5 inhibition in patients with diabetic cardiomyopathy. A total of 122 men and women (45 to 80 years) with long-duration (>3 years) and well-controlled type 2 diabetes mellitus (T2DM; HbA1c < 86 mmol/mol) were selected according to echocardiographic signs of cardiac remodeling. Patients were randomly assigned (1:1) to placebo or oral tadalafil (20 mg, once daily). The primary outcome was to evaluate sex differences in cardiac torsion change. Secondary outcomes were changes in cardiovascular, metabolic, immune, and renal function. At 20 weeks, the treatment-by-sex interaction documented an improvement in cardiac torsion (−3.40°, −5.96; −0.84, $P = 0.011$) and fiber shortening (−1.19%, −2.24; −0.14, $P = 0.027$) in men but not women. The primary outcome could not be explained by differences in cGMP concentrations or tadalafil pharmacodynamics. In both sexes, tadalafil improved hsa-miR-199-5p expression, biomarkers of cardiovascular remodeling, albuminuria, renal artery resistive index, and circulating Klotho concentrations. Immune cell profiling revealed an improvement in low-grade chronic inflammation: Classic $CD14^{++}CD16^{-}$ monocytes reduced, and $Tie2^{+}$ monocytes increased. Nine patients (14.5%) had minor adverse reactions after tadalafil administration. Continuous PDE5 inhibition could offer a strategy to target cardiorenal complications of T2DM, with sex- and tissue-specific responses. Further studies are needed to confirm Klotho and hsa-miR-199-5p as markers for T2DM complications.

INTRODUCTION

The prevalence of type 2 diabetes mellitus (T2DM) is increasing in both sexes. Cardiovascular diseases (CVDs) cause more than 75% of hospitalizations and 50% of deaths (*1*). One-third of patients with diabetes suffers from diabetic nephropathy (DN), the leading cause of end-stage renal disease. Common to cardiac and renal complications of T2DM is low-grade chronic inflammation, now recognized among the most important causes of death in the world (*2*).

Diabetic cardiomyopathy (DC) is a cluster of structural and functional changes in the hearts of patients with diabetes that develop independently of coronary events, hypertension, or cardiac valve disease (*3*). According to the American Heart Association (*4*) and the European Society of Cardiology (*5*), DC is characterized by a continuous spectrum of alterations. The asymptomatic first stage involves increased stiffness leading to reduced left ventricle (LV) compliance, impaired early diastolic filling, and prolonged isovolumetric relaxation with elevated LV end diastolic pressures (*6*). The second stage involves worsening of LV hypertrophy and diastolic dysfunction with the consequent development of heart failure with preserved ejection fraction (HFpEF) (*7*). As the disease progresses, the diastolic and the coexisting systolic dysfunctions lead to heart failure with reduced ejection fraction (HFrEF). Prolonged pre-ejection, shortened ejection, and increased filling resistance and pressures progressively worsen with higher heart failure (HF) stages. EF should not, therefore, be regarded as the hallmark of DC. Diastolic dysfunctions associated with compensatory LV concentric remodeling are often the only early alterations (*8*), with DN being the highest risk factor independently associated with preclinical worsening of systolic function (strain) (*9*). Despite proper glycemic control, DC still progresses with a broad spectrum of changes in extracellular matrix dynamics, cardiomyocyte signaling pathways, and proinflammatory triggers (*10*). The modulation of the nitric oxide (NO)–cyclic guanosine monophosphate (cGMP) pathway has gained attention because of its involvement in mitigating cardiac stress responses (*11*). Stimulation of the NO-cGMP pathway via phosphodiesterase 5 (PDE5) inhibition improves hypertrophy and cardiac damage from various injuries (*12*), conditions in which PDE5 is found overexpressed (*12*). However, there is no unanimous consensus on whether PDE5 inhibition (PDE5i) exerts direct or indirect

[1]Department of Experimental Medicine, Sapienza University of Rome, 00161 Rome, Italy. [2]Neuroendocrinology, Neuromed Institute, IRCCS, 86077 Pozzilli (IS), Italy. [3]Department of Cardiovascular and Respiratory Diseases, Sapienza University of Rome, 00161 Rome, Italy. [4]Department of Translational and Precision Medicine, Sapienza University of Rome, 00161 Rome, Italy. [5]Department of Radiological Sciences, Oncology and Pathology, Sapienza University of Rome, 00161 Rome, Italy. [6]Unit of Endocrinology and Diabetes, Department of Medicine, University Campus Bio-Medico di Roma, 00161 Rome, Italy. [7]Medical Statistics and Information Technology, AFaR, Fatebenefratelli Hospital, 00161 Rome, Italy. [8]Department of Life and Environmental Sciences, University of Cagliari, 09124 Cagliari, Italy.
*Corresponding author. Email: andrea.isidori@uniroma1.it (A.M.I.); daniele.gianfrilli@uniroma1.it (D.G.)
†These authors contributed equally to this work.
‡These authors contributed equally to this work as co–senior authors.

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cardioprotective effects and which are the cellular targets or underlying molecular mechanisms (*12*–*19*).

Some clinical trials assessing cardiac response to PDE5i showed conflicting results (*8*, *20*, *21*), and only two randomized clinical trials (RCTs) addressed the outcome of chronic PDE5i in DN (*22*, *23*). Early studies in men with HFpEF showed an improvement in cardiac geometry and systolic and diastolic function (*21*). The largest clinical trial on the effect of PDE5i on HFpEF (*20*), the RELAX trial, showed no beneficial effect of a 6-month escalating dose of daily PDE5i (sildenafil) in a mixed population (men and women) with a constellation of comorbidities, as opposed to antiremodeling effects observed in a homogeneous cohort of men with diabetes receiving a similar treatment (*8*). The two RCTs on DN enrolled only men, or two-thirds men, and showed a microalbuminuria reduction (*22*, *23*). Two independent meta-analyses confirmed that daily PDE5i administration improved cardiac function in patients with CVD, with excellent cardiovascular safety and tolerability (*24*, *25*).

Nondiabetic women have fewer cardiovascular events than same-aged men; however, HF becomes meaningfully greater in diabetic women than men (*1*), particularly in the presence of HFpEF (*26*, *27*). Preclinical (*28*) and clinical evidence (*1*) support a sex difference in vascular T2DM complications, and experimental mouse models demonstrate that PDE5i provides sex-specific cardioprotection (*29*). Such discrepancies increase the perception that PDE5i is effective in the presence of specific molecular pathways triggered by diabetes differently in men and women. In this study, we investigated whether continuous inhibition of PDE5 in patients with diabetes exerts sex-specific effects on cardiac contraction. We then explored metabolic, renal, and inflammatory pathways that could mediate cardioprotection.

RESULTS

From May 2014 to July 2018, 220 subjects were screened (Fig. 1): 96 [53 women (W) and 43 men (M)] were excluded (70 did not meet entry criteria, and 26 refused to participate), and 124 entered the study (57 W and 67 M). Sixty-two patients were randomly assigned to tadalafil and 62 to placebo. At baseline, two male patients (one in each group) were excluded after myocardial ischemia diagnosis, giving a final intention-to-treat (ITT) population of 122 (Table 1, baseline characteristics). The dropout rate was 13.1% (eight) for the tadalafil group and 9.8% (six) for placebo. All patients were European Caucasian, except two of Asian ethnicity. Concomitant disease and medications are listed in Table 1.

The study population consisted of middle-aged men and women (65 years, 60;69), with long-duration diabetes (13 years, 7;19), well controlled on antidiabetic therapies. All women were postmenopausal (67 years, [64;71]), as confirmed by low serum estrogens (estradiol, 7.9 pg/ml; 5;10); 38 men had testosterone (T) concentrations in the normal range for adults as defined by a $T \geq 3.5$ ng/ml (4.41 ng/ml, [3.98;5.82]), whereas the remaining 24 had testosterone in the low range of normal (3.08 nM, [2.7;3.2]). Cardiac magnetic resonance (CMR) baseline characteristics revealed a thickened septum, increased torsion (normal value between 10° and 15°) (*30*), and reduced strain (normal value, ≤14%) (*31*) in both groups, consistent with typical features of DC remodeling (Table 1). Two-thirds of study population was on antihypertensive medication, and 16% had DN.

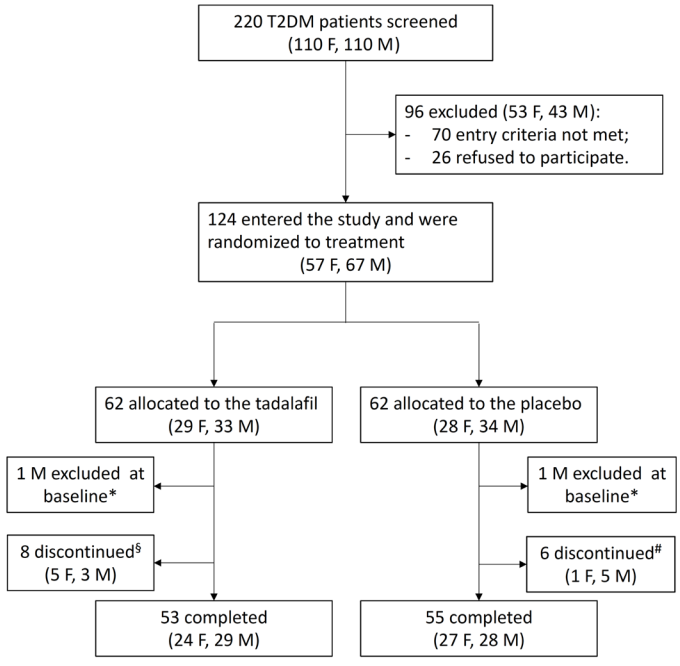

Fig. 1. Study flowchart (CONSORT). M, men; W, women. *Excluded for baseline cardiac resonance features of myocardial ischemia. §Seven patients were lost at follow-up; one discontinued due to stomachache. #Five patients were lost at follow-up; one patient had lung tumor diagnosed.

Primary outcome: LV torsion

Baseline cardiac torsion values were not different between groups ($P = 0.888$). Compared to placebo, 20-week tadalafil reduced cardiac LV torsion in men [estimated treatment difference (ETD) of −3.32°, −6.22 to −0.43, $P = 0.026$] but not women (ETD of 1.18°, −0.90 to 3.27, $P = 0.257$), providing a significant treatment-by-sex interaction for cardiac LV torsion ($P = 0.012$) with a mean torsion difference of −3.40° (−5.96 to −0.84, $P = 0.011$) between sexes (Table 2). Because all men were eugonadal and all women menopausal, gonadal status was a substantial determinant for the primary outcome. However, in men, absolute sex steroid concentrations did not contribute any further to the treatment-related change in LV torsion (see also the paragraph of the influence of gonadal status below).

LV strain, geometry, function, and contraction dynamics

Compared to placebo, tadalafil improved strain in men (ETD of −1.19%, −2.24 to −0.14, $P = 0.027$) but not women (ETD of −0.32%, −0.78 to 1.42, $P = 0.558$). Significant treatment-by-sex interaction was found ($P = 0.047$), with a difference in the shortening improvement of −1.26% (−2.35 to −0.17, $P = 0.025$) between sexes (Table 2). No changes were measured in LV mass index (LVMi), end diastolic volume index (EDVi), concentricity index (LVMi/EDVi), EF, and cardiac HF circulating marker NT-proBNP (Table 3). Both twist and untwist phases of cardiac contractions changed without sex-related differences (Tables 3 and 4): Compared to placebo, tadalafil reduced the time to peak (ETD of −16.18 ms, −30.70 to −1.67, $P = 0.028$) and the twist coefficient b_t (ETD of −0.05, −0.09 to −0.02, $P = 0.005$) while increasing the untwist coefficient b_{ut} (ETD of 0.06, 0.01 to 0.10, $P = 0.009$) and the recoil rate (ETD of 10.39%, 4.43 to 16.35, $P < 0.001$). T1 mapping at CMR revealed a significant treatment-related effect

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Table 1. Baseline characteristics of the full analysis set. Values are expressed as means (lower upper limit of 95% CI), median [25th;75th percentile], count (*n*), or percentages (%) as appropriate. BMI, body mass index; HbA1c, glycated hemoglobin; HOMA-IR, Homeostasis Model Assessment–Insulin Resistance; eGFR, estimated glomerular filtration rate; TEM, Tie2-expressing monocytes; NK, natural killer; FGF, fibroblast growth factor; SDMA, symmetric dimethylarginine; TGF, transforming growth factor; GLP-1, glucagon-like peptide; DPP, dipeptidyl peptidase; ACE, angiotensin-converting enzyme; ARBs, angiotensin receptor blockers.

	Total (*n* = 122)	Randomized placebo (*n* = 61)	Randomized tadalafil (*n* = 61)
Men *n* (%)	65 (53.3)	33 (54.1)	32 (52.5)
Testosterone (ng/ml)	4.0 [3.2;4.6]	4.0 [2.9;4.6]	3.7 [3.2;4.3]
Women *n* (%)	57 (46.7)	28 (45.9)	29 (47.5)
Estradiol (pg/ml)	7.9 (5.0;8.4)	5.0 (5.0;5.0)	7.4 (5.0;10.2)
Race			
Caucasian *n* (%)	120 (98.4)	60 (98.4)	60 (98.4)
Asian *n* (%)	2 (1.6)	1 (1.6)	1 (1.6)
Age (years)	65 [60;69]	66 [64;73]	65 [60;68]
Diabetes duration (years)	13 [7;19]	13 [7;18]	14 [6;20]
Body weight (kg)	80 (15.6)	78 (15.1)	82 (15.4)
BMI (kg/m^2)	28.53 [25.09;31.81]	26.96 [24.82;31.25]	29.11 [25.65;33.12]
Waist (cm)	102 [93;113]	101 [92;110]	104 [92;114]
Mean arterial pressure (mmHg)	91.16 (89.28;93.04)	92.32 (89.55;95.09)	90.67 (87.96;93.37)
Heart rate (bpm)	70 [66;77]	70 [64;77]	72 [67;77]
HbA1c (mmol/mol)	51 [45;60]	51 [44;58]	51 [45;61]
HOMA-IR	5.2 [2.3;6.5]	5.3 [2.6;5.9]	5.1 [2.2;7.4]
eGFR (ml/min/1.73m^2)	87.20 (73.60;95.10)	86.40 (69.10;93.60)	91.20 (73.80;95.75)
Cardiac morpho-functional and kinetic parameters			
Left ventricular torsion (°)	17.81 (10.75;27.44)	17.72 (10.16;27.03)	17.76 (11.07;27.61)
Left ventricular strain (%)	−13.78 (−18.14;−10.25)	−13.97 (−18.50;−10.62)	−13.67 (−18.14;−9.49)
Left ventricular mass index (LVMi; g/m^2)	84.50 (72.75;99.87)	81.95 (79.40;90.45)	86.60 (74.80;108.40)
Left ventricular end diastolic volume index (EDVi; ml/m^2)	57.45 (49.05;65.32)	55.55 (46.05;62.67)	60.50 (50.70;66.30)
Concentricity index (LVMi/EDVi)	1.48 (1.27;1.84)	1.47 (1.19;1.85)	1.50 (1.29;1.80)
Ejection fraction (%)	64.40 [58.00;68.42]	63.85 [56.77;68.60]	65.07 [58.50;68.20]
Interventricular septum (mm)	11.00 (10.00;13.00)	11.00 (9.00;13.00)	11.00 (10.00;13.00)
T rise max (°)	31.66 [25.10;44.51]	31.27 [28.38;43.21]	32.42 [23.54;48.85]
b_t rise coefficient	0.13 (0.09;0.20)	0.13 (0.09;0.20)	0.13 (0.08;0.22)
T_0 decay (°)	18.85 (14.97;22.65)	19.07 (15.03;22.78)	18.01 (14.92;22.45)
b_{ut} recoil coefficient	0.11 [0.08;0.17]	0.12 [0.08;0.18]	0.10 [0.07;0.15]
Time to peak (ms)	125.00 (125.00;150.00)	125.00 (100.00;150.00)	125.00 (125.00;150.00)
Recoil rate (%)	30.32 [18.82;42.96]	31.30 [26.58;44.84]	26.54 [14.88;38.06]
T1 mapping - extracellular volume	26.18 [24.93;27.00]	26.24 [25.05;27.06]	26.26 [24.76;27.61]
Immunological profile (cell/μl)			
CD14$^+$ monocytes	597 (444;776)	615 (500;789)	596 (432;778)
CD14^{++} CD16^{neg} (classical) monocytes	445 (350;614)	468 (365;662)	444 (358;604)
CD14^{++} CD16$^+$ (intermediate) monocytes	18 (8;35)	17(7;35)	24 (9;39)
CD14$^+$ CD16^{++} (nonclassical) monocytes	49 (28;93)	60 (29;107)	45 (25;77)

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	Total ($n = 122$)	Randomized placebo ($n = 61$)	Randomized tadalafil ($n = 61$)
TEMs	18 (9;27)	19 (11;33)	15 (7;26)
$CD3^+$ lymphocytes	1078 (415;1994)	1056 (320;1899)	1099 (432;2220)
T $CD8^+$ lymphocytes	310 (207;486)	282 (215;570)	318 (200;444)
T $CD8^+$ $CD4^+$ lymphocytes	49 (37;98)	60 (42;111)	46 (34;110)
T $CD4^+$ lymphocytes	651 (140;1390)	646 (138;1400)	652 (69;1678)
B $CD19^+$ lymphocytes	226 (133;355)	250 (124;388)	188 (137;267)
$CD56^+$ $CD3^-$ NK cells	237 (131;390)	243 (134;410)	216 (131;391)
$CD56^+$ $CD3^-$ $CD16^+$ NK cells	147 (62;258)	170 (63;268)	119 (58;260)
Circulating markers			
Klotho (pg/ml)	71.9 (66.82;86.22)	73.70 (68.50;86.50)	69.90 (65.65;87.05)
FGF-23 (pg/ml)	34.61 [29.12;189.66]	31.62 [28.94;81.53]	58.94 [29.54;214.29]
SDMA (μg/ml)	233.56 [201.58;244.30]	231.08 [185.17;243.51]	235.64 [215.31;244.75]
TGF-β (ng/ml)	32.49 (21.99;44.89)	32.82 (21.26;44.89)	31.10 (22.56;44.75)
Titin (pg/ml)	95.33 [93.51;99.32]	95.85 [93.26;97.71]	95.21[93.58;101;14]
NT-proBNP (pg/ml)	86.29 (29.83;109.35)	79.15 (28.51;101.20)	94.39 (31.23;112.15)
hsa-miR-199-5p (copies/μl × 10^5)	0.30 [0.06;2.67]	0.44 [0.01;2.29]	0.30 [0.10;4.29]
cGMP (pmol/ml)	1.1 [1.05;1.20]	1.10 [1.08;1.20]	1.05 [0.97;1.2]
Comorbidities, *n* (%)			
Retinopathy	13 (10.7)	7 (11.5)	6 (9.8)
Neuropathy	20 (16.4)	6 (9.8)	14 (22.9)
Nephropathy	21 (16.4)	9 (14.7)	12 (19.7)
Asymptomatic carotid atherosclerotic disease	22 (18.0)	9 (14.7)	13 (21.3)
Smoking habit	23 (18.8)	11 (18.0)	12 (19.7)
Medications, *n* (%)			
Diabetes			
Metformin	92 (75.4)	44 (72.1)	48 (78.7)
Sulfonylureas, *n* (%)	28 (23.0)	14 (22.9)	14 (22.9)
GLP-1 agonists, *n* (%)	4 (3.3)	3 (4.9)	1 (1.6)
DPP-IV inhibitors, *n* (%)	22 (18.0)	12 (19.7)	10 (16.4)
Gliflozins, *n* (%)	4 (3.3)	2 (3.3)	2 (3.3)
Insulin, *n* (%)	16 (13.1)	7 (11.5)	9 (14.7)
Lipid-lowering drugs, *n* (%)	67 (54.9)	36 (59.0)	31 (50.8)
Statin, *n* (%)	48 (40.0)	30 (49.2)	18 (29.5)
Antihypertensives, *n* (%)	70 (57.4)	33 (54.1)	37 (60.7)
ACE inhibitors, *n* (%)	29 (23.8)	11 (18.0)	18 (29.5)
ARBs, *n* (%)	36 (33.6)	20 (32.8)	16 (26.2)
Calcium blockers, *n* (%)	25 (29.5)	10 (16.4)	15 (24.6)
Diuretics, *n* (%)	22 (18.0)	11 (18.0)	11 (18.0)
β-Blockers, *n* (%)	30 (24.6)	8 (13.1)	22 (36.1)
Cardioaspirin, *n* (%)	42 (34.4)	20 (32.8)	22 (36.1)
Other antiplatelet, *n* (%)	9 (7.4)	3 (4.9)	6 (9.8)
Anticoagulants, *n* (%)	4 (3.3)	2 (3.3)	2 (3.3)

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Table 2. Primary analysis: Change in left ventricular torsion and strain. ANOVA, values are expressed as mean (lower upper limit of 95% CI). Boldfaced *P* value are significant.

Sex	Δ Change from baseline, placebo	Within group *P*	Δ Change from baseline, tadalafil	Within group *P*	Treatment effect	Between group *P*
Left ventricular torsion (°)						
All	0.49 (−0.71;1.69)	0.401	−0.58 (−1.83;0.68)	0.563	−1.07 (−2.80;0.66)	0.223
Women	−0.06 (−1.78;1.66)	0.931	1.13 (−0.55;2.80)	0.161	1.18 (−0.90;3.27)	0.257
Men	1.05 (−1.63;2.72)	0.296	−2.28 (−4.14;−0.41)	**0.042**	−3.32 (−6.22;−0.43)	**0.026**
Treatment-by-sex interaction	1.11 (−1.32;3.53)	0.362	−3.40 (−5.96;−0.84)	**0.011**		**0.012**
Left ventricular strain (%)						
All	0.19 (−0.31;0.70)	0.232	−0.24 (−0.80;0.31)	0.077	−0.44 (−1.18;0.31)	0.249
Women	0.07 (−0.66;0.79)	0.864	0.39 (−0.37;1.15)	0.299	−0.32 (−0.78;1.42)	0.558
Men	0.32 (−0.39;1.03)	0.377	−0.87 (−1.68;−0.07)	**0.031**	−1.19 (−2.24;−0.14)	**0.027**
Treatment-by-sex interaction	0.25 (−0.79;1.30)	0.628	−1.26 (−2.35;−0.17)	**0.025**		**0.047**

in cardiac extracellular volume (ECV; ETD of 0.74, 0.04 to 1.44, $P = 0.037$), sustained by declining ECV in the placebo group (Table 3).

Hemodynamic effects

A treatment-related reduction was observed in mean heart rate (−3.77 bpm, −7.50 to −0.04; $P = 0.046$; Table 3 and Fig. 2A). The same was also observed in mean arterial pressure (MAP; −3.89 mmHg, −7.30 to −0.49; $P = 0.026$) but only when baseline MAP was included as covariate in the analysis of covariance (ANCOVA) model. However, on randomization, there was no difference in blood pressures (BP) between the treatment arms (ΔMAP 2.22 mmHg, −1.40 to 5.85, $P = 0.227$) or between men and women (M versus W differences in MAP: $\Delta MAP_{placebo}$ −0.75 mmHg, −6.02 to 4.51, $P = 0.77$; $\Delta MAP_{tadalafil}$ −0.41 mmHg, −5.64 to 4.83, $P = 0.870$). Therefore, the model was run without covariate correction and showed no treatment-related (−2.48 mmHg, −6.82 to 1.86; $P = 0.259$; Table 3) or sex-related differences ($P = 0.373$; Table 4). Analyses on cardiac outcomes yielded similar findings when corrected or not for MAP (treatment-by-sex interaction for cardiac torsion and strain: $P = 0.015$ and $P = 0.038$, respectively). To check the variations in BP over the 5-month treatment period, a repeated-measure analysis was performed including BP values at baseline and 3 and 5 months. The analysis revealed no significant difference (time-by-treatment interaction $P = 0.654$; table S1 and fig. S1). Overall, the antihypertensive effect of chronic tadalafil, if any, could not account for the observed findings.

Circulating biomarkers

Analysis of circulating cardiac markers showed treatment-related reduction in serum titin concentrations (Fig. 2B), sustained by a progressive rise in the placebo group that was prevented by tadalafil (ETD of −4.87 pg/ml, −9.28 to −0.46, $P = 0.029$; Table 3), but no change was found for angiopoietin 1 and 2 (table S2). Hsa-miR-199-5p is a small noncoding RNA expressed in cardiomyocytes and endothelial cells and up-regulated in cardiac hypertrophy or ischemic injury. Hsa-miR-199-5p was significantly reduced by tadalafil (ETD of −3.53 copies/μl × 10^5, −6.39 to −0.67, $P = 0.015$) (Table 3 and fig. S2), and its modulation strongly inversely correlates with changes in circulating Klotho ($r^2 = -0.65$, $P = 0.001$) but not titin concentrations. Treatment-by-sex interactions for secondary outcomes are shown in Table 4 and table S3.

Metabolic outcomes

No major metabolic changes were observed in the study. Glucose metabolism remained unchanged: The estimated treatment effects for glycated hemoglobin (HbA1c) and Homeostasis Model Assessment–Insulin Resistance (HOMA-IR) were −1.00 (−3.31 to 1.32, $P = 0.390$) and 0.11 (−1.63 to 1.85, $P = 0.900$), respectively (Table 3 and Fig. 2, C and D). Mirroring the effects on glucose metabolism, tadalafil did not affect lipid homeostasis. Similarly, body composition did not change as demonstrated by body mass index (BMI), waist, and dual-energy x-ray absorptiometry (DEXA) visceral and truncal adipose tissue (table S2).

Immune outcomes

Treatment did not affect total circulating white blood cells, lymphocytes, basophils, eosinophils, or neutrophils. Fluorescence-activated cell sorting (FACS) analysis of peripheral blood mononuclear cell subsets revealed significant reduction of total $CD14^+$ and classic $CD14^{++}CD16^-$ monocytes with ETD of −216 cells/μl (−338 to −94; $P = 0.001$; Fig. 2E) and −159 cells/μl, respectively (−246 to −71; $P < 0.001$) (Table 3). The number of Tie2-expressing monocytes (TEMs) increased (Fig. 2F) as a result of treatment, with an ETD of 19 cells/μl (12 to 25, $P < 0.001$) in both sexes, although showing a greater response in men (26 cells/μl, 17 to 35) than women (11 cells/μl, 2 to 20), and treatment-by-sex interaction of $P = 0.018$ (Table 4 and fig. S3). Treatment-related effects were found in circulating transforming growth factor–β (TGF-β) (ETD of −14.18 ng/ml, −20.58 to −7.77, $P < 0.001$; Fig. 2G) but not other chemokines such as monocyte chemoattractant protein-1 and soluble Tie2 (table S2).

Renal outcomes

Renal hemodynamic parameters were significantly modulated by tadalafil treatment, with a 3.96% reduction in the renal resistive index (RRI) (−7.60 to −0.32, $P = 0.032$) (Table 3 and Fig. 2H) and with no changes in estimated glomerular filtration rate (eGFR; table S2). Among patients presenting with albuminuria at baseline ($n = 20$),

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Table 3. Analysis of secondary outcomes. UACR, urinary albumin-to-creatinine ratio; cGMP, cyclic guanosine monophosphate. Boldfaced *P* value are significant.

Outcome measures	Δ Change from baseline, placebo	Within group *P*	Δ Change from baseline, tadalafil	Within group, *P*	Treatment effect	Between group, *P*
Left ventricular mass index (LVMi; g/m^2)*	2.46 (−1.74;6.65)	0.246	1.29 (−3.20;5.78)	0.568	−1.17 (−7.13;4.97)	0.700
Left ventricular end diastolic volume index (EDVi; ml/m^2)	2.47 (−0.32;5.26)	0.082	3.19 (−0.21;6.18)	0.051	0.73 (−3.36;4.81)	0.721
Concentricity index (LVMi/EDVi)	−0.05 (−0.16;0.06)	0.367	−0.05 (−0.16;0.07)	0.389	0.01 (−0.15;0.17)	0.901
Ejection fraction (EF %)	0.41 (−2.03;2.86)	0.739	−0.92 (−2.45;0.60)	0.230	−1.29 (−4.26;1.68)	0.387
NT-proBNP	−8.83 (−22.39;4.73)	0.172	5.73 (−9.81;21,27)	0.428	14.56 (−6.06;35.18)	0.163
Time to peak (ms)	11.87 (2.00;21.75)	**0.020**	−4.31 (−14.95;6.33)	0.422	−16.18 (−30.70;−1.67)	**0.028**
b_t*	0.01 (−0.01;0.03)	0.322	−0.04 (−0.07;−0.02)	**0.002**	−0.05 (−0.09;−0.02)	**0.005**
b_{ut}	-0.02 (−0.05;0.01)	0.188	0.04 (0.01;0.07)	**0.010**	0.06 (0.01;0.10)	**0.009**
Recoil rate (%)*	−3.95 (−8.00;−0.10)	0.056	6.44 (2.15;10.73)	**0.004**	10.39 (4.43;16.35)	**<0.001**
Extracellular volume (ECV)*	−0.78 (−1.27;−0.29)	**0.002**	−0.04 (−0.55;0.47)	0.876	0.74 (0.04;1.44)	**0.037**
Heart rate (bpm)	0.71 (−1.84;3.25)	0.579	−3.06 (−5.79;−0.33)	**0.029**	−3.77 (−7.50;−0.04)	**0.046**
Mean arterial pressure (MAP; mmHg)	−1.20 (−4.16; 1.75)	0.437	−3.68 (−6.86;−0.51)	**0.027**	−2.48 (−6.82;1.86)	**0.259**
Titin (pg/ml)	5.30 (2.35;8.25)	**0.001**	0.43 (−2.85;3.71)	0.794	−4.87 (−9.28;−0.46)	**0.029**
hsa-miR-199-5p (copies/μl × 10^5)	0.69 (−1.25;2.62)	0.479	−2.84 (−4.95;−0.73)	**0.010**	−3.53 (−6.39;−0.67)	**0.015**
HbA1c (nmol/mol)	−0.90 (−2.48;0.68)	0.260	−1.90 (−3.59;0.21)	0.051	−1.00 (−3.31;1.32)	0.390
HOMA-IR	−0.16 (−1.44;1.11)	0.803	−0.05 (−1.23;1.13)	0.933	0.11 (−1.63;1.85)	0.900
CD14$^+$ total monocytes (cells/μl)	−65 (−148;16)	0.119	−281 (−372;−192)	**<0.001**	−216 (−338;−94)	**0.001**
CD14$^+$CD16$^-$ classical monocytes (cells/μl)	−72 (−131;−14)	**0.017**	−231 (−296;−167)	**<0.001**	−159 (−246;−71)	**<0.001**
TEM (cells/μl)	8 (3;12)	**0.001**	28 (22;31)	**<0.001**	19 (12;25)	**<0.001**
TGF-β (ng/ml)	−4.30 (−8.91;0.30)	0.067	−18.49 (−22.94;−14.03)	**<0.001**	−14.18 (−20.58;−7.77)	**<0.001**
Renal resistive index (%)	1.88 (−0.48; 4.24)	0.117	−2.08 (−4.85;0.69)	0.139	−3.96 (−7.60;−0.32)	**0.032**
Albuminuria ($log_{mg/24hours}$)†	0.13 (−0.01;0.27)	0.089	−0.22 (−0.35;−0.10)	**0.001**	−0.35 (−0.54;−0.16)	**0.001**
Albuminuria (% from baseline)	49.93 (12.32;87.53)	0.069	−36.03 (−67.56;−4.50)	**0.008**	−85.96 (−135.03;−36.88)	**0.002**
UACR (mg/g)†	191 (−118;499)	0.399	−184(−409;42)	0.125	−374 (−756; 7)	0.056
Klotho (pg/ml)*	−6.10 (−19.85; 7.65)	0.379	33.12 (17.59;48.66)	**0.001**	39.22 (18.31;60.13)	**<0.001**
SDMA (μg/ml)	48.82(33.25;64.38)	**<0.001**	7.43 (−10.42;25.28)	0.409	−41.39 (−65.07;−17.70)	**0.001**
BMI (kg/m^2)	−0.63 (−1.56;0.30)	0.181	−0.06 (−1.01;0.90)	0.900	−0.57 (−0.76;1.90)	0.393
cGMP (pmol/ml)	−0.03 (−0.09; 0.04)	0.360	0.10 (0.01;0.19)	**0.031**	0.13 (0.01;0.24)	**0.026**

*Baseline outcome measure included in the model as covariate; †Only patients with albuminuria at baseline ($n = 20$); values are expressed as means (lower upper limit of 95% CI).

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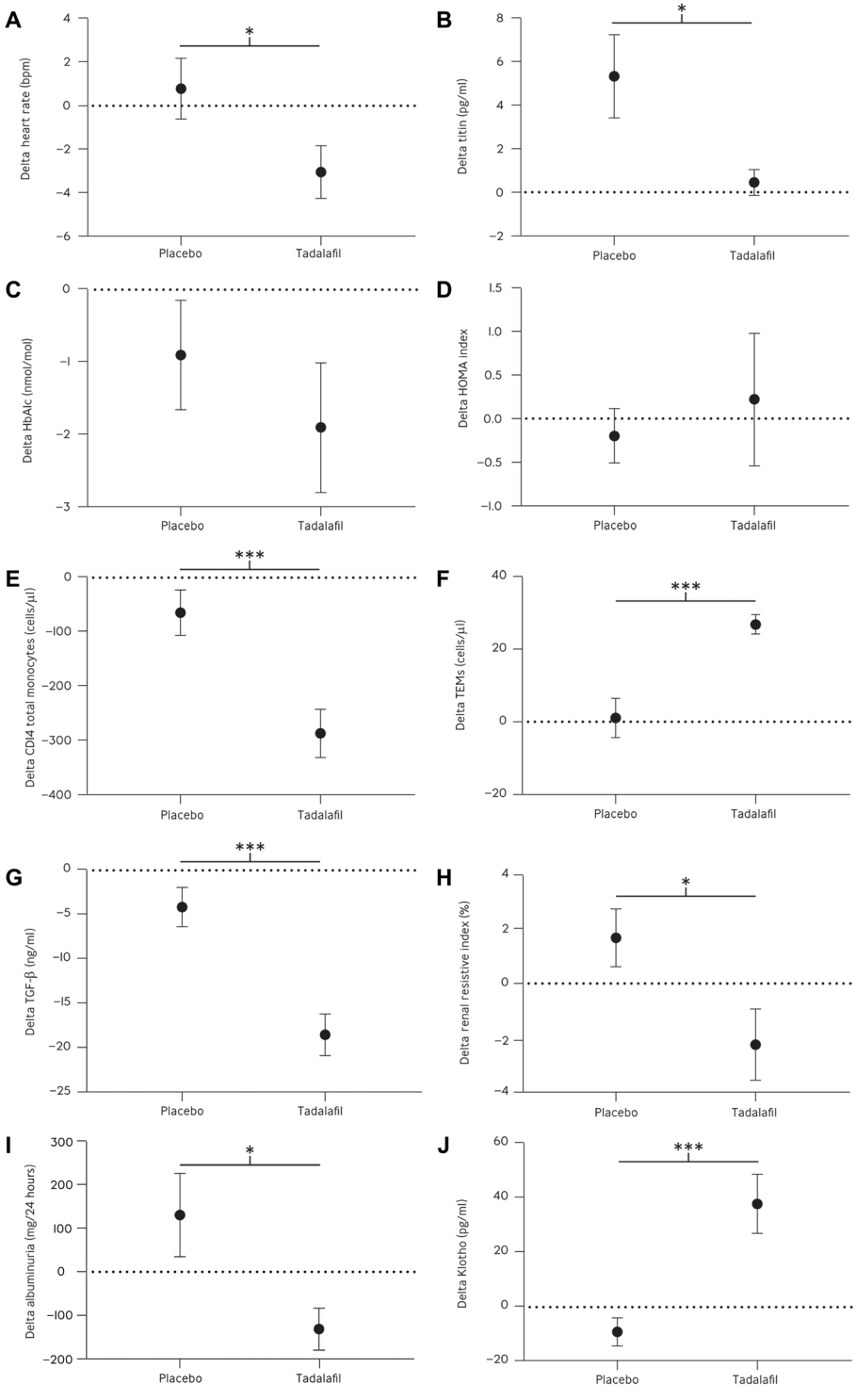

Fig. 2. Summary of the delta of secondary outcome measures in placebo and tadalafil groups. (**A**) Delta change in heart rate (beats per minute; placebo, 54 of 55; tadalafil, 47 of 53). (**B**) Delta change in circulating titin concentrations (picograms per milliliter; placebo, 43 of 55; tadalafil, 35 of 53). (**C**) Delta change in circulating HbA1c (millimoles per mole; placebo, 55 of 55; tadalafil, 48 of 53). (**D**) Delta change in HOMA index (placebo, 21 of 55; tadalafil, 23 of 53). (**E**) Delta change in circulating CD14 total monocytes (cells per microliter; placebo, 54 of 55; tadalafil, 45 of 53). (**F**) Delta change in circulating TEMs (cells per microliter; placebo, 54 of 55; tadalafil, 48 of 53). (**G**) Delta change in circulating TGF-β concentrations (nanograms per milliliter; placebo, 39 of 55; tadalafil, 41 of 53). (**H**) Delta change in RRI (%) measured by Doppler ultrasound (placebo, 34 of 55; tadalafil, 24 of 53). (**I**) Delta change in 24-hour urinary albuminuria (only patients with albuminuria at baseline) (milligrams per 24 hours; placebo, 8 of 55; tadalafil, 12 of 53). (**J**) Delta change in circulating Klotho concentrations (picograms per milliliter; placebo, 43 of 55; tadalafil, 34 of 53). Data are expressed as delta means and SEM between the assessments (5 months minus baseline). Differences between deltas were analyzed by ANOVA test. *$P < 0.05$ and ***$P < 0.001$.

tadalafil reduced the 24-hour albuminuria: ETD of −257.42 mg/24 hours (−492.25 to −22.58, $P = 0.034$; Fig. 2I) corresponding to −36.03% (−67.56 to −4.50) compared to placebo (49.93%, 12.32 to 87.53, $P = 0.002$; Table 3). The same trend was observed when the analysis was performed for urinary albumin-to-creatinine ratio (UACR) (ETD of −374 mg/g, −756 to 7, $P = 0.056$; Table 3). Among normoalbuminuric patients, albuminuria developed in 5 of 43 (11.6%) patients randomized to placebo but not in the 38 taking tadalafil.

Serum concentrations of Klotho and symmetric dimethylarginine (SDMA), both predictors of renal impairment in DN (*32*, *33*), were modulated by the treatment, with an ETD of 39.22 pg/ml (18.31 to 60.13, $P < 0.001$; Fig. 2J) and −41.39 µg/ml (−65.07 to −17.70, $P < 0.001$), respectively (Table 3). The treatment modulation of Klotho showed borderline association with reduction in albuminuria ($r = -0.22$, $P = 0.061$). Serum calcium, phosphate, and the co-receptor of Klotho, fibroblast growth factor 23 (FGF-23), remained unchanged (table S2).

cGMP and tadalafil plasma concentrations

There was no intergroup difference in baseline cGMP concentrations ($P = 0.07$). A treatment-related increase in plasma cGMP concentrations was observed in the tadalafil group (0.13 pmol/ml, 0.01 to 0.24; $P = 0.026$) without sex differences ($P = 0.233$) (Tables 3 and 4). A repeated-measure analysis was performed to check for possible differences in tadalafil pharmacokinetics (Fig. 3). Drug concentrations were undetectable at the baseline in both study arms and at all subsequent evaluations in patients receiving placebo. Conversely, patients randomized to the drug had tadalafil concentrations in the therapeutic range at both 3 and 5 months (Fig. 3A). Tadalafil concentrations increased and plateaued at 3 months ($tadalafil_{men}$ of 94.8 µg/liter; 57.3 to 125.8 versus $tadalafil_{women}$ of 97.3 µg/liter; 66.3 to 132.3, $P = 0.465$), remaining stable at 5 months ($tadalafil_{men}$ of 94.5 µg/liter; 74.5 to 129.5 versus $tadalafil_{women}$ of 130.8 µg/liter; 80.0 to 189.9, $P = 0.437$)

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Table 4. Summary of treatment-by-sex interaction. See also table S3. Boldfaced *P* value are significant.

Outcomes	Treatment-by-sex interaction *P*
Left ventricular torsion	**0.012**
Left ventricular strain	**0.047**
b_t	0.184
b_{ut}	0.417
Time to peak	0.249
Recoil rate	0.078
Extracellular volume (ECV)	0.249
Heart rate	0.453
Mean arterial pressure	0.373
Titin	0.797
hsa-miR-199-5p	0.194
$CD14^+$ total monocytes	0.469
$CD14^+CD16^-$ classical monocytes	0.245
TEM	**0.018**
TGF-β	0.209
Renal resistive index	0.773
Albuminuria	0.809
Klotho	0.470
SDMA	0.306
cGMP	0.233
Tadalafil	0.534

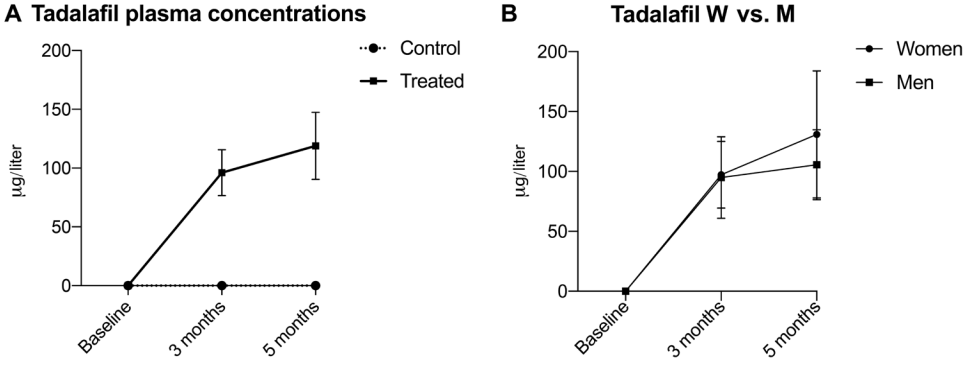

Fig. 3. Tadalafil plasma concentrations throughout the study time frame. (**A**) Tadalafil plasma concentrations in patients assigned to placebo (10 of 55; black dots) and tadalafil (18 of 53; black squares) at baseline and 3 and 5 months after randomization. (**B**) Sex differences in tadalafil plasma concentrations in patients randomized to tadalafil. Black dots, women (five placebo and nine tadalafil); black squares, men (five placebo and nine tadalafil; time-by-sex interaction $P = 0.534$). Data are expressed as means and SEM and were analyzed by one-way repeated-measures ANOVA test.

similarly in both sexes (time-by-sex interaction $P = 0.534$; Fig. 3B). Taking into account the possible effects of CYP3A4 activity on tadalafil concentrations (*34*), we corrected the analysis for statin use, but no difference in tadalafil plasma concentrations was observed between statin users and nonusers at 3 months ($tadalafil_{users}$ of 98.7 µg/liter; 74.6 to 122.8 versus $tadalafil_{nonusers}$ of 93.9 µg/liter; 72.3 to 115.5, $P = 0.330$) and 5 months ($tadalafil_{users}$ of 125.5 µg/liter; 89.3 to 161.7 versus $tadalafil_{nonusers}$ of 102.5 µg/liter; 70.1 to 134.8, $P = 0.474$; time-by-statin use interaction $P = 0.533$).

Influence of gonadal status on the primary outcome

High-sensitivity measurement of testosterone, free testosterone, and dehydroepiandrosterone and its sulfate was carried out by liquid chromatography with tandem mass spectrometry (LC-MS/MS; see table S4) (*35*). Baseline androgen concentrations were not directly correlated with treatment-related changes in cardiac torsion or strain (table S5). When stratified by gonadal status, tadalafil reduced cardiac LV torsion in men with higher testosterone concentrations (ETD of −3.61°, −6.08 to −1.14, $P = 0.006$) but not in those with testosterone in the lower range (ETD of 0.92°, −2.91 to 4.75, $P = 0.240$) with a mean torsion difference of −4.53° (−8.84 to −0.22, $P = 0.041$) between groups. Subgroup analysis in women was not performed because they were all postmenopausal. Baseline characteristics of the full analysis set stratified by sex, as well as analysis of the secondary outcomes stratified by sex, are shown in tables S6 and S7.

Effects of concomitant medication on primary outcome and biomarkers

Hsa-miR-199-5p can be induced by β-adrenergic signaling (*36*). Because of the imbalance of patients receiving β-blockers between the treatment groups (13.1% versus 36.1%, $P = 0.002$), we corrected the analysis of variance (ANOVA) for β-blocker use and found the following: no difference in baseline hsa-miR-199-5p concentrations between groups ($mir199\text{-}5_{placebo}$ of 0.44 copies/µl × 10^5, 0.01 to 2.29; $mir\text{-}199\text{-}5p_{tadalafil}$ of 0.30 copies/µl × 10^5, 0.10 to 4.29; $P = 0.414$), no effect on delta torsion ($P = 0.451$), and no interaction of β-blockers by treatment ($P = 0.467$) or β-blockers by treatment by sex ($P = 0.352$). Conversely, the treatment-by-sex interaction for the primary outcome remained significant ($P = 0.014$), and the marginal differences were substantially confirmed (−2.97°; 95% CI: −0.33, −5.62 in men; +1.1°; 95% CI: −1.42, +3.58 in women), excluding a confounding effect of β-blocker use. Similar results were obtained for hsa-miR-199-5p: The main effect of β-blocker on delta hsa-miR-199-5p was not significant ($P = 0.495$), and neither were the β-blocker–by–treatment ($P = 0.790$) or β-blocker–by–treatment–by–sex interactions ($P = 0.974$), whereas the effect of treatment on hsa-miR-199-5p remained significant ($P = 0.049$) and the marginal differences confirmed (−3.42 copies/µl; 95% CI: −6.82, −0.02).

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Fewer patients in the placebo group received angiotensin-converting enzyme (ACE) inhibitors, and therefore, an adjustment for baseline covariate imbalance was also performed for this drug class. The main effect on delta torsion from baseline was not significant ($P = 0.622$), and neither was the double interaction of ACE inhibitors by treatment ($P = 0.614$) or the triple interaction of ACE inhibitors by treatment by sex ($P = 0.900$). However, the interaction of treatment by sex remained significant ($P = 0.048$), and the marginal differences were substantially confirmed (−3.56°; 95% CI: −6.86, −0.27 in men; 0.73°; 95% CI: −2.03, 3.49 in women).

Safety

Fourteen minor adverse reactions to tadalafil (4 M and 10 W) were recorded (table S8). Of the study patients, nine (14.7%) had myalgia (one M and eight W), six (9.8%) had gastric reflux (three M and three W), four (6.5%) had headache (one M and three W), and two (3.3%) had flushing (one M and one W). Myalgia was significantly more frequent in women receiving tadalafil compared to men or those receiving placebo [odds ratio (OR) of 14.00, 1.60 to 122.33, $P = 0.017$]. No events required hospital admission, and one patient (male) in the tadalafil group withdrew because of stomachache.

DISCUSSION

Our study investigated sex differences in cardiac response to daily PDE5i administration in patients with DC. Twenty weeks of tadalafil improved cardiac contraction kinetics in men, but not postmenopausal women, by reducing torsion and improving shortening. Combining CMR tagging and T1 mapping studies with measurements of several biomarkers of cardiovascular, immune, and renal function, our trial revealed that tadalafil ameliorated renal hemodynamics and filtration, lowered inflammation, and improved markers of cardiac remodeling, namely, Klotho, $Tie2^+$ monocytes, titin, and hsa-miR-199-5p expression.

In DC, parameters of contraction kinetics, torsion, and strain change precociously (*10*) and have been demonstrated to have a prognostic role for HF, cardiovascular events, and mortality (*37*), making them useful to test prevention strategies. Cardiac torsion is a compensatory mechanism triggered by hypertrophic ventricular remodeling, and strain is a sensible measure of contraction efficiency that becomes impaired before EF declines in DC. A large systematic review found that any unit of increase in standardized torsion is associated with a 1.6 times greater reduction in mortality compared to change in LVEF (*38*). We documented a one standardized increase in strain and 0.9 reduction in torsion in men when compared to women, pointing toward a clinically meaningful sex-specific intramyocardial effect of continuous PDE5i in reversing maladaptive T2DM cardiac remodeling.

Several trials investigated the putative effects of PDE5i on cardiac and renal function. The inconsistencies in their findings originate from the heterogeneity of study cohorts with respect to underlying conditions (pulmonary arterial hypertension, HFpEF, and HFrEF), their severity (concomitant renal impairment or diabetes mellitus), type of PDE5i and dose regimen used, and ignoring sex differences (table S9). Restricting the analysis only to RCTs including patients with HFpEF, cardioprotective effects were described in four trials evaluating PDE5i efficacy on ventricular function. In 2011, Guazzi and colleagues (*21*) published the first human evidence of improvement of LV diastolic function and cardiac geometry after chronic (12 months) PDE5i in 44 patients with HFpEF, but only men were evaluated by echocardiography. Our 2012 study (*8*) described the beneficial effect of sildenafil on cardiac contraction kinetics and dynamics in 59 men with DC, but this trial was limited by including only men, having a short treatment duration, and not performing T1 mapping.

The 2013 larger multicenter RELAX trial studied 216 male and female patients with HFpEF treated with sildenafil and found no significant improvement in exercise capacity or clinical status (*20*). RELAX was the largest male-female study to report CMR, but analysis by sex was not performed; the study was heterogeneous, and patients had important comorbidities and noncardiac factors that could have influenced the primary outcome. Patients had mild diastolic dysfunction, only half met the left ventricular hypertrophy (LVH) criteria, and half of the patients did not complete the post-treatment CMR evaluation. Because PDE5 expression and protein kinase G (PKG) signaling increase in hypertrophic injuries and sildenafil is more effective in LVH, the RELAX trial failure to reach statistical significance is likely due to the above listed study characteristics. Last, in 2017, Liu and colleagues (*39*) found no differences in LV geometry, systolic and diastolic function, or renal function in 52 patients with symptomatic HFpEF treated with sildenafil (60 mg) three times daily for 12 weeks. Again, 71% of the patients enrolled were postmenopausal women, but analysis by sex was not performed.

Sex differences in CVD are recognized (*40*), but sexual dimorphism is neglected in clinical trial design, pointing to an urgent need to analyze data by sex rather than adjusted for sex. Studies have described sex differences in response to tadalafil for pulmonary hypertension (*41*). Estrogens slow the progression toward cardiac hypertrophy (*42*), whereas hyperglycemia can disrupt estrogen signaling and impair endothelium-dependent relaxation and NO production. This attenuates female cardiovascular protection, putting women at greater risk than men (*43*).

Although not designed to address treatment-by-sex interactions, the data in RELAX table S3 show an opposite trend in the primary outcome (VO_2 per minute) between sexes: sildenafil-treated men showed a tendency toward improvement, but women and placebo controls did not. Our trial documented similar treatment effects, with men improving and women worsening, achieving statistical significance for cardiac torsion and strain. Most preclinical animal studies showing cardioprotection of PDE5i only examined males. The few studies that included females revealed that intramyocyte effects of sildenafil are estrogen dependent (*29*) and mediated by the non-nuclear signaling through the estrogen receptor-α (*44*). On the other hand, the favorable effects of testosterone in mediating PDE5 expression, NO generation, and response to PDE5i are well established in men (*45*, *46*). We hypothesize that gonadal status might shape how males and females respond to PDE5i, supporting the rationale for a hormone replacement therapy to ensure PDE5i effects. Similarly, a meta-analysis on 34,000 patients with diabetes demonstrated that major adverse cardiovascular event (MACE) reduction is significantly lower in women than in men receiving sodium-glucose transport protein 2 inhibitors (SGLT2is), whereas MACE is similar between sexes if receiving glucagon-like peptide-1 receptor agonists (GLP-1RAs) (*47*). Unfortunately, a subanalysis by estrogen status was not performed. Different PDE5i pharmacokinetics between men and women, or CYP450 enzyme activity, could also be responsible for the heterogeneity in outcomes. However, most

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previous data analyses on sex were obtained post hoc (*48*), which yielded conflicting results. In contrast, our prospective study was specifically designed to explore the role of sex rather than merely adjusting for it, revealing a more complex scenario with sex-related responses in some but not all outcomes. Our work further showed no sex-related differences in tadalafil plasma concentrations at any study time point, including patients receiving statins, thus providing additional safety data for diabetic patients while confirming other smaller and shorter studies on tadalafil plasma distribution and metabolism (*49*, *50*).

Various mechanisms have been proposed for PDE5i cardiovascular protection through cardiomyocytes and endothelium-driven, metabolic, immunoregulatory, or indirect renal effects (*51*), although unequivocal identification of the main cell target has not been achieved. Several groups have detected cardiac PDE5, albeit at a very low expression, in cardiomyocytes under physiological conditions (*52*, *53*). PDE5 expression was also found up-regulated in dysfunctional hearts, to a varying degree (*54*). Different PDE5 expression according to the underlying condition may justify, at least in part, the controversial results on PDE5i use in cardiovascular disorders. Pioneering work by Takimoto *et al.* (*12*) demonstrated that PDE5i reverses and PDE5 overexpression in cardiomyocytes promotes transverse aortic constriction (TAC)–induced cardiac remodeling through the cGMP/PKG pathway (*55*, *56*). In the same year, another study concluded that PKG depleted from noncardiac cells is not sufficient to exacerbate hypertrophy (*14*), challenging the previous findings. A genetic mouse model of broadly expressed or cardiomyocyte-restricted activated PKG displays LV hypertrophy, suggesting that PKG signaling has a detrimental role in cardiac hypertrophy and fibrosis (*17*, *57*). Others have reported that sildenafil does not protect against TAC-induced cardiac remodeling and LV function deterioration in mice expressing mutant PKG (unable to bind effectors) in contrast to wild-type controls, again pointing to a key role for PKG in antiremodeling effects of cGMP (*15*). Although these studies recognize some cardioprotection from PDE5i, they do not unequivocally attribute it to a direct intracardiomyocyte effect, rather suggesting an indirect endothelial effect.

We used a combination of T1 mapping and innovative biomarkers to investigate cardiac tissue changes. ECV decreased in the placebo but not in the tadalafil group. ECV increases with late-stage DC due to myocardial fibrosis, but low ECV values have been described in cardiac hypertrophy without fibrosis (*58*). In our cohort, tadalafil prevented changes in ECV; however, the 1% observed change is not expected to be clinically relevant.

Previous studies demonstrated that PDE5i treatment increased titin phosphorylation, enhancing diastolic distensibility (*59*). In line with this, our results showed that tadalafil reduced the twisting coefficient (b_t) and time to maximum peak of torsion during systole while increasing the untwisting coefficient (b_{ut}) and recoil rate during diastole, meaning better diastolic compliance. However, we found a sex-independent effect for cleaved titin, resulting in higher circulating serum titin in placebo-treated subjects. Titin cleavage results from a complex balance between metalloproteinases (MMPs) and their inhibitors, which are altered in diabetes (*60*). The expression of MMP type 2, colocalizing with titin in the sarcomere, is inhibited by PDE5i (*61*, *62*). The sex difference in MMP expression, higher in men compared to premenopausal women, is lost after menopause (*63*). This might account for the lack of treatment-by-sex interaction in titin cleavage, as compared to the effects on strain and torsion.

Sex-independent changes observed in the absolute count of circulating hsa-miR-199-5p are consistent with the systemic effects of PDE5i. Mature microRNAs (miRNAs) are well-recognized key regulators of cardiovascular function and influence the RNA-induced silencing complex to suppress gene expression or promote its degradation. Hsa-miR-199-5p regulates vascular growth, primarily angiogenesis, in response to arterial occlusion and is elevated in patients with coronary or peripheral artery disease (*64*, *65*). Preclinical studies revealed that increased expression of hsa-miR-199-5p disrupts cardiomyocytes' sarcomere structure. Cardiomyocytes become elongated, resemble a phenotype of eccentric cardiac hypertrophy (*66*), inhibit cardiomyocyte autophagy, and impair endothelial cell function (*67*). A large LVH study showed that hsa-miR-199-5p correlates well with hypertrophy but not fibrosis (*68*). This supports our findings of increased LVMi but no fibrosis at T1 mapping. The down-regulation of hsa-miR-199-5p, for example, by insulin receptor activation of Akt, is sufficient to induce hypoxia-inducible factor 1 and Sirt1 (*36*). In T2DM, insulin sensitivity is reduced, and consequently, the Akt-dependent cardioprotection is missing. In the CECSID trial, we confirmed a sildenafil-induced increase in circulating Sirt1 (*69*). Conversely, β-adrenergic receptor activation induces hsa-miR-199-5p. Consistent with preclinical observations that sildenafil counteracts β-adrenergic action with in vivo and in vitro mouse models (*52*), the current trial finds a treatment-related effect on heart rate. PDE5i activates PKG and enhances Akt phosphorylation (*70*); β-adrenergic stimulation antagonizes insulin, impairs glucose uptake, and inhibits the protective effect of insulin in cardiomyocytes (*71*). We believe that T2DM and insulin resistance impair Akt signaling and lead to increased hsa-miR-199-5p associated with reduced Sirt1 and Klotho. PDE5i might help restore this pathway at cardiomyocyte, endothelial, or both levels.

When proper glycemic control is achieved, T2DM is associated with persistent low-grade inflammation, higher number of circulating classical monocytes, M1 macrophage polarization (*72*), and increased TGF-β (*60*). The T2DM immune profile disrupts endothelial repair, compromising cardiac integrity. We found that tadalafil reduced $CD14^+$ and classic $CD14^{++}CD16^-$ monocytes, restored TEMs (a subset of M2 monocytes), and played a crucial role in tissue repair by attenuating the proinflammatory cell profile and the profibrotic TGF-β pathways. Experimental modulation of hsa-miR-199-5p regulates perfusion recovery and collateral arteriogenesis by monocyte trafficking (*64*). Thus, PDE5i not only mutes hypertrophic signaling within cardiomyocytes but also prevents overreaction of the immune system, favoring a balanced tissue healing. Whether this is a direct effect on immune cells or exerted through an endothelium-dependent regulation of immune cell trafficking remains unclear.

Hypertension is one of the major abnormalities in HFpEF (*73*); when treated, diastolic function improves (*74*). However, the marginal reduction induced by tadalafil (3.68 mmHg), albeit significant, cannot be regarded as physiologically relevant because a 5- to 10-mmHg drop is considered clinically meaningful (*75*–*79*). Moreover, multiple analyses demonstrated the independence of cardiac and renal treatment–related changes from BP variations in our study: (i) Tadalafil's effects on torsion and strain were sex dependent, as opposed to the effects on MAP (sex independent); (ii) the adjustment for MAP did not affect cardiac and renal outcomes; and (iii) the repeated-measures analysis did not find meaningful BP variation throughout the study period. Together, this suggests that the observed BP changes are more likely physiological fluctuations than true responses to treatment.

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In preclinical studies, we showed that PDE5i treatment slowed the progression of DN and reduced albuminuria by improving RRI (*16*) and restored podocyte function through cGMP–PKG–PPAR-γ (peroxisome proliferator–activated receptor–γ)–dependent inhibition of calcium transient receptor potential channel 6 (*18*). In the present trial, we measured significant improvement in DN as a secondary outcome. Tadalafil reduced intraglomerular hemodynamics (RRI) and albuminuria (−36%) in patients who had it at baseline, changes in line with the effects of combined RAS inhibitors in DN (*80*). Eleven per cent of placebo-treated patients progressed toward an albuminuric state, compared to patients not taking tadalafil. SDMA serum concentrations showed similar changes, suggesting that tadalafil could halt DN progression (*81*). Change in albuminuria is now considered a biologically plausible surrogate endpoint for chronic kidney disease (CKD) progression. Increased albuminuria has been shown to be a strong predictor of end-stage CKD (*82*, *83*), and our findings are consistent with trials reporting a reduction in the UACR as primary (*22*, *23*) or secondary outcomes (*84*).

We described a pharmacological modulation of α-Klotho. α-Klotho is the obligate co-receptor of FGF-23 and maintains phosphate and calcium homeostasis, but it has several independent activities. Its concentrations decrease early in DN progression (*85*), correlate with an increased risk of HF (*86*), and have been suggested as a useful prognostic tool and therapeutic target for CVD (*87*). The α-Klotho gene contains a noncanonical PPAR-responsive element, and tadalafil activates the PPAR-γ pathway through PKG-cGMP dependence (*18*). Tadalafil could also increase α-Klotho through down-regulation of hsa-miR-199-5p, which is up-regulated in cardiac hypertrophy (*66*–*68*) and when the renal filtration barrier is disrupted (*88*). Studies showed that miR-199 down-regulation increases Klotho and improves microalbuminuria in DN (*88*). Our data demonstrate that the treatment-related increases in Klotho in both sexes correlate with the suppression of circulating hsa-miR-199-5p. Unlike the intramyocardial effects of PDE5i, which depend on estrogen signaling (*29*), hsa-miR-199 expression and modulation have been demonstrated to be estrogen independent (*89*). This could explain why, in our cohort, renal, but not cardiac, outcomes were sex independent. Last, enhancing α-Klotho concentrations has been shown to reduce renal inflammation in db/db mice (*90*) and prevent monocytes from adopting inflammatory-like phenotypes (*91*), consistent with the changes that we observed in classical monocytes.

Limitations

Because of the sample size and length, our no-profit, spontaneous trial could only measure putative differences in cardiac contraction kinetics (i.e., torsion and strain), which are not regarded as established surrogates for cardiovascular outcomes in clinical practice (such as MACE). However, evidence demonstrating the ability of such early markers to predict mortality and hospitalization in patients with unexplained HF symptoms is accumulating, and use of such markers is gaining momentum (*92*). Recent trials on cardiovascular safety from GLP-1 RAs and SGLT2is proved a reduction in MACE in patients with established atherosclerotic CVD (*93*), independent of glucose control. However, it is important to acknowledge that recommendations for the use of GLP-1 RAs and SGLT2is are mainly for the secondary prevention of CAD and based on evidence from subgroup analyses of trials including patients with and without T2DM (*94*). Our cohort included subjects with earlier stages of cardiovascular impairment, so MACE was not chosen as an outcome. A control cohort of healthy subjects and patients affected by HFpEF, without diabetes, could have compared DC with other cardiac damage models or normal hearts. A control group with diabetic postmenopausal women under hormone replacement therapy could reveal what proportion of the observed findings was related to sex or gonadal status, clarifying estrogen's role in PDE5i's efficacy on DC. However, menopausal status is a physiological condition, and very few diabetic women are taking estrogens over the age of 60, therefore not limiting the clinical applicability of our findings. Last, tissue samples could have provided further insights into the molecular mechanisms of hsa-miR-199-5p and Klotho regulation.

Conclusions

This trial clarifies major inconsistencies about the cardioprotective role of PDE5i in T2DM, reconciling experimental and clinical evidence. Continuous PDE5i has a beneficial effect on maladaptive cardiac kinetic changes triggered by diabetes in men but not in women. Conversely, tadalafil modulation of the miR-199-5p–α-Klotho axis appears as sex independent: The reduction of hsa-miR-199-5p results in increased α-Klotho and improved low-grade inflammation, halting renal disease progression in both sexes. On the basis of past and present findings, continuous PDE5i can hamper diabetic microvascular disease (albuminuria, erectile dysfunction, and myocardial remodeling) in men and protect women at risk for DN, through mechanisms that are more complex than originally thought, likely involving different molecular players at different sites. When compared to other pharmacological strategies that are mainly effective as secondary prevention strategies, the cost of cardiorenal protection with PDE5i is much lower and acting through a mechanism that is independent of (and therefore additive to) metabolic control. By demonstrating tissue-sex–specific effects of PDE5i, this trial suggests new molecular pathways targeted by PDE5i, raising the intriguing idea that they may be indicated for the prevention of cardiac remodeling and renal complication in T2DM.

MATERIALS AND METHODS

Study design

We performed a 20-week, double-blind (patient/outcome assessor), randomized, placebo-controlled interventional trial (NCT01803828) in outpatient clinic patients. Inclusion criteria were T2DM of duration of >3 years, age range of 45 to 80 years, BMI of <40 kg/m^2, and HbA1c of <86 mmol/mol. Exclusion criteria included uncontrolled hypertension, PDE5i use, congenital or valvular cardiomyopathy, history of ischemic heart disease, chronic or acute atrial fibrillation, proliferative retinopathy, contraindications to tadalafil treatment or contrast CMR imaging, hospitalization, or acute inflammatory/ischemic events within the previous 6 months. Eligible patients were screened with standard two-dimensional echocardiography and selected according to the features of early-stage DC: early cardiac remodeling (interventricular septum, ≥11 mm in men and ≥10 mm in women) or diastolic dysfunction (E/A < 1 or E/e′ > 10 at pulsed-wave-Doppler and tissue Doppler) with normal EF (EF > 50%) (*6*, *7*).

Our previous study (*8*) demonstrated significant change in cardiac torsion (in degrees) in men treated with sildenafil for 3 months. We observed a decrease in the sildenafil arm (Δ = −3.89°, SD = 3.11°) and an increase in the placebo arm (Δ = +2.13°, SD = 2.35°) (comparison: 6.02°; 95% CI: 4.49–7.54). We hypothesized that the treatment effect for men in the present study would have a 90% power of recognizing statistical significance (with alpha level) in effect size f = 0.33 for

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treatment-by-sex interaction. The *f* value for women anticipated an increase equal to that for men in the placebo arm (+2.13°) but a null effect in the active arm (0.0°). This scenario, assuming a homogeneous within-cell SD of 3.0, results in an *f* value of 0.32, corresponding to an eta-squared value of 0.095 for treatment-by-sex interaction. On the basis of these assumptions, 105 patients should be recruited, raised to 124 to account for a dropout rate of 15%. Last, 62 men and 62 women should be recruited and randomly assigned 1:1 to the two-treatment arms.

We conducted the trial between May 2014 and January 2019 (Fig. 1) according to CONSORT 2010 guidelines. Patients signed informed consent and were randomized to daily placebo or selective PDE5i (tadalafil, 20 mg/day) for 20 weeks. The study was performed in the Policlinico Umberto I in Rome (Italy) and approved by the local ethical review board (3083/14), published on public registries (NCT01803828, EUDRACT 2014-000077-39), and conducted under the Declaration of Helsinki and good clinical practice. Full protocol was published online (*95*).

Randomization and masking

Data were anonymized using a three-digit code, and patients were randomly assigned (1:1) to identical placebo or tadalafil 20-mg pills (Cialis, Lilly, Utrecht). A computer-generated random sequence stratified randomized patients by diabetes duration (years) and HbA1c. The trial was double-blind for patients, medical team, and magnetic resonance imaging (MRI) assessors.

Procedures

Patients were instructed to take the assigned medication after breakfast with water. Patients' blood samples were collected between 8:00 and 9:00 a.m., after an overnight fast. Changes were not allowed in the placebo/tadalafil dose/timing, and other medications were not permitted except for intercurrent illness (after consultation with the study team). Patients and controls were on an antidiabetic diet; diets were assessed, and diabetic nutritional recommendations were provided. Study patients underwent blood sampling, CMR, renal Doppler ultrasonography with measurements of kidney volumes and RRI, whole-body DEXA scans, diabetic neuropathy evaluation, and blood sampling for secondary outcome measures (see below).

Outcomes

The primary outcome was to measure the sex difference in cardiac torsion change from baseline to week 20, assessed by CMR with myocardial tagging. Secondary outcomes were treatment-related differences from baseline to week 20 in (i) cardiac shortening (strain), LV geometry (LVMi; EDVi; concentricity index = LVMi/EDVi), and cardiac performance (EF); (ii) myocardial fibrosis assessed by T1 mapping at MRI; (iii) metabolic assessments (HbA1c, HOMA-IR, and lipid profile); (iv) circulating profibrotic, proinflammatory, and proangiogenic chemokines; (v) profile of circulating immune cells; (vi) markers of DN and renovascular function (eGFR and albuminuria in a 24-hour urine collection and RRI measured by color Doppler ultrasonography); and (vii) body composition assessed by DEXA scan. Evidence suggests the Klotho pathway as a link between cardiac, renal, and immunological impairment, so we amended the protocol to add Klotho and related has-miR-199-5p as secondary outcomes. Secondary outcomes were tested for sex differences in treatment-related effects.

We will present results on the other prespecified secondary outcomes (i.e., diabetic neuropathy, questionnaires' scores, and profibrotic and proinflammatory cytokines) in a separate report. Albuminuria was defined as ≥30 mg of albumin in the 24-hour urine collection. Drug adherence was assessed by patients' self-reports and doctors' pill counts. Study safety was monitored and included adverse events, hospital admission, requests for medical assistance, laboratory analyses, and vital signs. An independent local data monitoring committee recorded adverse events, routine blood biochemistry, and blood counts but were not otherwise involved in the trial.

CMR imaging

CMR imaging studies were performed in a paired fashion (i.e., baseline and follow-up for each patient, examined one after the other) by an experienced radiologist (N.G.) who was blinded to the treatment allocation. A 1.5-T scanner (Avanto, Siemens, Healthcare Solutions) with eight-element phased-array coil and electrocardiogram triggering was used. The standard CMR protocol was previously described (*10*). To determine the ECV fraction, T1 mapping was performed using a modified Look-Locker inversion recovery sequence before and 15 min after injection of contrast agent; as previously described (*96*), late gadolinium-enhanced imaging was performed between 10 and 15 min after contrast agent injection (0.2 mmol/kg of gadoterate meglumine; Guerbet) to detect focal areas of myocardial fibrosis and rule out previous myocardial infarction. CMR studies were analyzed using dedicated software (cvi42 version 5.3, Circle Cardiovascular Imaging), as reported (*97*). Interobserver analyses were performed by an experienced radiologist (M.F.) for 10% of cases. A Likert scale was used to assess image quality for each component (2 = good or no major artifact; 1 = adequate or only minor artifact; 0 = poor or major artifact). Scans with a Likert scale of 0 were not analyzed. Kinetics and indexes of cardiac geometric deformation were analyzed as previously explained (*8*, *10*). Global torsion of the LV (θ, in degrees) was calculated as the difference between the clockwise rotation (positive) of the apex and the counterclockwise rotation of the base: $\theta = \theta_{apex} - \theta_{base}$.

Laboratory investigation

Serum samples obtained from patients at baseline (time 0) and end (time +20 weeks) visits were collected and stored at −80°C. Serum amount of soluble TIE2 (Thermo Fisher Scientific), FGF-23 (MyBioSource), TGF-β (TGFβ-1) (Invitrogen), titin (MyBioSource), SDMA (Abcam), and Klotho (Biorbyt) was measured using an enzyme-linked immunosorbent assay (ELISA). Serum SHBG concentrations (EH421RB, Thermo Fisher Scientific) and plasma cGMP amount (catalog number ADI-900-164, Enzo Life Sciences, Farmingdale, NY) were measured using a complete ELISA kit as per the manufacturer's instructions. NT-proBNP was analyzed by electrochemiluminescence immunoassay on the Elecsys 1010/2010 System (Roche Diagnostics). FACS analysis was performed according to our protocol, as previously described (*19*).

Ultrahigh-pressure liquid chromatography analysis

Plasma samples for tadalafil measurements were collected at 24 (±2) hours after the last administration. The results were compared with the therapeutic range published by the regulatory authorities (*98*).

Sample preparation

Two hundred microliters of thawed plasma samples was dissolved in 200 μl of acetonitrile containing the internal standard sildenafil at 100 μg/ml. Samples were then vortexed and centrifuged for 5 min at 13,000 rpm. The supernatant was filtered with 0.22-μm PTFE

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syringe filters and injected for ultrahigh-pressure liquid chromatography (UHPLC) analysis.

UHPLC conditions

An Agilent Technologies 1290 LC was equipped with Kinetex 5-µm EVO C18 100 A, 150 mm by 2.1 µm column (Agilent Technologies, Palo Alto, CA) and maintained at 25°C in a thermostatted column compartment. Mobile phases consisted of deionized water containing 0.1% formic acid and 2 mM ammonium acetate (A) and acetonitrile containing 0.1% formic acid (B). An isocratic elution mode consisting of 55% A and 45% B was used. The mobile phase flow rate was set to 0.7 ml/min. The total chromatographic run time was 3.00 min.

MS/MS conditions

An Agilent Technologies 6460-QQQ mass spectrometer with a Jetstream electrospray source was operated in positive and negative ionization mode with the following parameters: nitrogen drying gas temperature at 350°C, nitrogen sheath gas temperature at 325°C, nitrogen drying gas flow at 7.0 liters/min, nitrogen sheath gas flow at 11.0 liters/min, nebulizer pressure of 3.45 bar, capillary voltage of 3500 V, and nozzle voltage of 1500 V. In the positive mode, the dynamic multiple reaction monitoring method monitored transitions of 390.4 > 268.3 (collision energy-CE = 16 V) for tadalafil and 475.3 > 283.3 (CE = 18 V) for sildenafil, respectively.

Absolute miRNA copy quantification

miRNA evaluation was performed by droplet digital polymerase chain reaction (ddPCR) that allows absolute nucleic acid quantification, with precision and reproducibility greater than that of traditional real-time PCR (Bio-Rad Laboratories) (*99*). Total RNA was extracted and retrotranscribed using the TaqMan miRNA Kit (Thermo Fisher Scientific, USA) and hsa-miR-199-5p–specific stem-loop primer (code: 4427975, assay ID 000498, Thermo Fisher Scientific). Amplification was carried out using 5 µl of cDNA template, 1 µl of TaqMan miR-199 probe, and ddPCR supermix (Bio-Rad Laboratories). The PCR mixture was loaded in a disposable droplet generator to create oil-emulsion droplets and transferred to a 96-well PCR plate, sealed, and quantified at endpoint in a QX200 Droplet Reader System using QuantaSoft software (Bio-Rad Laboratories). The fraction of positive droplets was quantified assuming a Poisson distribution. Samples were performed in triplicate, and concentration results were expressed as the number of copies of hsa-miR-199-5p per microliter per sample.

Statistical analysis

Efficacy analyses were based on an ITT approach that included data from all the randomized patients who had received at least one dose of the study drug, with analysis according to the randomization treatment. Baseline to week 20 changes were analyzed with an ANCOVA model that included baseline outcome as a covariate and treatment as a fixed effect and used the last observation-carried-forward principle. Treatment-by-time and treatment-by-sex interactions were investigated to assess sex differences and were interpreted as significant at $P < 0.05$ and possible for $0.05 \leq P < 0.10$. Standardized residuals were tested for normality using the Shapiro-Wilk test. Homoscedasticity and homogeneity of variances were assessed by visual inspection and Levene's test. Multicollinearity was assessed by variance inflation factor. The least squares mean estimates with 95% CI of the ETD (ETD: tadalafil versus placebo) were calculated using Bonferroni correction. For multiple comparisons among the several secondary outcomes, the Benjamini-Hochberg correction set at 5% of false discovery rate was used.

To describe MAP and tadalafil plasma concentration variations throughout the study, we retrospectively analyzed the 3-month visit for both variables. A one-way repeated-measures ANOVA was conducted to reveal any statistically significant differences over the study time frame. There were no outliers, and the data were normally distributed, as assessed by boxplot and Shapiro-Wilk test ($P > 0.05$). For tadalafil plasma concentrations, the assumption of sphericity was violated, as assessed by Mauchly's test of sphericity, $\chi^2 = 7.49$, $P = 0.024$. Therefore, a Huynh-Feldt correction was applied ($\varepsilon = 0.870$).

Discrete secondary endpoints were analyzed using χ^2 analysis or Fisher's exact test in cases of few events. ORs (tadalafil versus placebo) and 95% confidence intervals (CIs) were calculated using a logistic regression model. Data are presented as mean (lower bound; upper bound of 95% CI) or median (25th; 75th percentile) as appropriate for distribution. A two-sided $P < 0.05$ was regarded as significant. In case of significant treatment-by-sex interactions, post hoc analysis included differences between eugonadal men (testosterone concentrations of >3.54 ng/ml) and men with mild hypogonadism (testosterone concentrations between 2.3 and 3.54 ng/ml).

SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S3
Tables S1 to S9
MDAR Reproducibility Checklist
References (*100*–*113*)

View/request a protocol for this paper from *Bio-protocol*.

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Sex-specific effects of daily tadalafil on diabetic heart kinetics in RECOGITO, a randomized, double-blind, placebo-controlled trial

Riccardo PofiElisa GiannettaTiziana FeolaNicola GaleaFederica BarbagalloFederica CampoloRoberto BadagliaccaBiagio BarbanoFederica CiolinaGiuseppe DefeudisTiziana FilardiFranz SestiMarianna MinnettiCarmine D. VizzaPatrizio PasqualettiPierluigi CaboniIacopo CarboneMarco FranconeCarlo CatalanoPaolo PozzilliAndrea LenziMary Anna VenneriDaniele GianfrilliAndrea M. Isidori

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Conditional cardioprotection

Phosphodiesterase 5 (PDE5) inhibition has been shown to have inconsistent cardioprotective effects. Here, Pofi *et al.* conducted a small phase 4 trial to assess sex differences in cardiac remodeling in patients with diabetes and diabetic cardiomyopathy treated with the PDE5 inhibitor tadalafil. They observed an improvement in cardiac shortening and torsion in men but not women, whereas circulating has-miR-199-5p was reduced and Klotho increased with treatment in both sexes. Certain populations of monocytes were also altered with treatment. Results highlight the importance of considering sex-specific differences in treatment response.

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