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RESEARCH ARTICLE

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SIRT1 rs7896005 polymorphism affects major vascular outcomes, not all-cause mortality, in Caucasians with type 2 diabetes: A 13-year observational study

Angela Dardano[1] | **Daniela Lucchesi**[1] | **Monia Garofolo**[1] | **Elisa Gualdani**[2] | **Pierpaolo Falcetta**[1] | **Veronica Sancho Bornez**[1] | **Paolo Francesconi**[2] | **Stefano Del Prato**[1] | **Giuseppe Penno**[1]

[1]Section of Diabetes and Metabolic Disease, Department of Clinical and Experimental Medicine, University of Pisa and Azienda Ospedaliero-Universitaria Pisana, Pisa, Italy

[2]Epidemiology Unit, Regional Health Agency (ARS) of Tuscany, Florence, Italy

Correspondence

Stefano Del Prato, Department of Clinical and Experimental Medicine, Azienda Ospedaliero-Universitaria Pisana, University of Pisa, Via Piero Trivella 56124, Pisa, Italy.
Email: stefano.delprato@unipi.it

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Abstract

Aims: SIRT1 exerts effects on ageing and lifespan, as well cardiovascular (CV) disease risk. SIRT1 gene is very polymorph with a few tagging single nucleotide polymorphisms (SNPs) so far identified. Some SNPs, including rs7896005, were associated with type 2 diabetes (T2DM). We aimed to ascertain whether this SNP may be associated with CV disease at baseline as well with these same outcomes and all-cause mortality over a 13-year follow-up.

Materials and Methods: Genotypes of SIRT1 gene were determined using TaqMan SNP assay.

Results: Out of 905 T2DM, 9.1% had the AA genotype, 43.2% the AG, and 47.7% the GG. Hardy–Weinberg Equilibrium was met (minor allele frequency 0.306; $p = 0.8899$). At baseline, there was no difference across genotypes for sex, age, diabetes duration, CV risk factors, treatments, and microangiopathy. Major CV outcomes, myocardial infarction (MI), any coronary heart disease (CHD), and peripheral artery disease (PAD) were more frequent in GG than in AA/AG (p from 0.013 to 0.027), with no association with cerebrovascular events. By fully adjusted regression, GG remained independently related to major CV outcomes, MI, CHD, and PAD. Over follow-up, we recorded 258 major CV events (28.5%; AA/AG 25.2%, GG 32.2%; $p = 0.014$) with an adjusted hazard ratio (HR) of GG versus AA/AG of 1.296 (95% CI 1.007–1.668, $p = 0.044$); 169 coronary events (18.7%; AA/AG 15.4%,

Abbreviations: ACR, Albumin to Creatinine Ratio; AIR, Acute Insulin Response; ALT, Alanine Aminotransferase; ARS, Antioxidant Redox Signalling; AST, Aspartate Aminotransferase; BMI, Body Mass Index; BP, Blood Pressure; CAD, Coronary Artery Disease; CHD, Coronary Heart Disease; CI, Confidence Intervals; CKD, Chronic Kidney Disease; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; CVD, Cardiovascular Disease; DD, Diabetes Duration; DKD, Diabetic Kidney Disease; dBP, Diastolic Blood Pressure; eGFR, estimated GFR; ESRD, End Stage Renal Disease; GGT, Gamma-Glutamyl Transferase; HbA1c, Glycated Haemoglobin A1c; HDL-C, High-Density Lipoprotein Cholesterol; HR, Hazard Ratio; hs-CRP, High-sensitive C-Reactive Protein; IQR, Interquartile Range; K-M, Kaplan-Meier; LDL-C, Low-Density Lipoprotein Cholesterol; MAF, Minor Allele Frequency; MI, Myocardial Infarction; OR, Odds Ratio; PAD, Peripheral Artery Disease; PYs, Person-Years; RAS, Renin-Angiotensin System; ROS, Reactive Oxygen Species; SAPHIR, Salzburg Atherosclerosis Prevention Program in subjects at High Individual Risk; sBP, systolic Blood Pressure; SIR, Silent Information Regulator; SIRT, Sirtuin; SNP, Single Nucleotide Polymorphism; T2DM, Type 2 Diabetes Mellitus; UACR, Urine Albumin-to-Creatinine Ratio.

Angela Dardano and Daniela Lucchesi have contributed equally to the study.

GG 22.2%; $p = 0.006$) with HR 1.522 (1.113–2.080, $p = 0.008$); 79 (8.7%) hospitalisation for heart failure (AA/AG 7.0%, GG 10.6%; $p = 0.045$) and HR 1.457 (0.919–2.309, $p = 0.109$); 36 PAD (4.0%; AA/AG 2.3%, GG 5.8%; $p = 0.007$) with HR 2.225 (1.057–4.684, $p = 0.035$). No association was found with cerebrovascular events, end stage renal disease, and all-cause mortality.

Conclusions: The rs7896005 SNP of SIRT1 might play a role in cardiovascular disease, mainly CHD risk in T2DM. Results call for larger association studies as well as studies to ascertain mechanisms by which this variant confers increased risk.

KEYWORDS
all-cause mortality, cardiovascular outcomes, observational study, SIRT1 gene, type 2 diabetes

1 | INTRODUCTION

Silent information regulator genes include an across-species highly conserved family of proteins (sirtuins) that represent a complex response system affecting several biological aspects of ageing, longevity, and diseases.[1] Seven sirtuin genes (*SIRT1* through *SIRT7*) have been identified in mammals, *SIRT1* being the first discovered. These genes share essential functions as dynamic regulators of other genes and they code for seven distinct sirtuin enzymes that act as nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases or mono-ADP-ribosyltransferases. Sirtuins might be specific for different tissues, cellular localisation, enzymatic activities, and molecular targets.[2] As such sirtuins are believed to contribute to regulating nutrient sensing and utilisation, metabolic rate, energy homoeostasis and, ultimately, metabolic disease.[3] Moreover, sirtuins have been shown to be involved in a wide range of physiological and pathological processes, including age-related disorders, energy responses to low calorie availability, stress resistance, apoptosis, and inflammation.[3]

SIRT1 is located in the nucleus and the cytoplasm and it is the mammalian homologue of the yeast SIR2. In consideration of its effects on cell cycle, differentiation, senescence, apoptosis, and mitochondria metabolism, it is the most studied sirtuin.[4] *SIRT1*, known as a longevity gene, protects cells against oxidative and genotoxic stress, promotes DNA stability, contrasts inflammation, and participates in the regulation of energy homoeostasis as well as lipid and glucose metabolism.[5,6] These effects are largely mediated via interaction with protein substrates in many signalling pathways including the forkhead-box O transcription factors family, nuclear factor kappa B (NF-kB), peroxisome proliferator-activated receptor gamma-assisted activating factor-1 (PGC-1), nuclear factor erythroid2-related factor 2 (Nrf2), and tumour suppressor p53.[4]

Single nucleotide polymorphisms (SNPs) of the *SIRT1* gene have been shown to be associated with reduced acute insulin secretion in response to i.v. glucose and increased risk for type 2 diabetes (T2DM; rs10509291 and rs7896005) in Pima Indians,[6] to T2DM-related traits (rs7896005) in Mexicans,[7] and to insulin resistance and increased risk for T2DM (rs10509291 and rs10823112) in a Chinese Han population.[8] Furthermore, an interactive effect of an *SIRT1* promoter region polymorphism (rs12778366) on T2DM susceptibility has been described in the North Indian population,[9] while two different SNPs, rs7895833 and rs1467568, have been claimed to be involved in foetal programming during malnutrition, thus affecting T2DM risk later in life.[10]

SIRT1 is a modifier of human life expectancy. Indeed, SIRT1 affects long-term-survival modulating lifespan[11–13] though results are inconclusive[14] or controversial.[15–17] *SIRT1* is also likely to contribute to cardiovascular (CV) integrity[18] most likely through an anti-atherogenic effect in endothelial and vascular smooth muscle cells (VSMCs) as well as macrophages,[19–22] raising the hypothesis that genetic variants of the *SIRT1* gene may play a role in individual CV risk as suggested by initial studies.[23–26]

The present study extends the only report so far available in T2DM[26] by investigating the association between the rs7896005 SNP of *SIRT1* with the risk of CV disease in individuals with T2DM in a cross-sectional as well as 13-year prospective analysis.

2 | MATERIALS AND METHODS

2.1 | Study design and participants

A total of 961 T2DM individuals were consecutively enroled amongst those attending the Diabetes Outpatient Clinic of the Azienda Ospedaliero Universitaria Pisana between 1 November 2002 and 30 April 2004. For the purpose of this study, male or female subjects aged ≥18 years and <75 years, with T2DM (based on World Health Organization criteria) were recruited upon providing voluntary written informed consent. Pregnant women, individuals of non-white ethnicity, those with type 1 diabetes, and those on dialysis or with renal transplantation were excluded. The cross-sectional and prospective analysis were approved by the local Ethics Committee. The flow-chart of the study is shown in Figure S1.

Information about onset and duration of diabetes, smoking habits, current glucose-lowering treatments, and concomitant blood pressure (BP)- and lipid-lowering, and anti-platelet therapies were

recorded for each participant along with measurement of body weight and height (for calculation of body mass index [BMI]), waist circumference, and BP. Hypertension was defined as systolic BP > 140 mmHg and/or diastolic BP > 90 mmHg and/or use of any BP-lowering agent. Finally, a blood sample was obtained after an overnight fast for determination of plasma glucose level, glycated haemoglobin A1c (HbA1c), lipid profile, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl-transferase (GGT), uric acid, fibrinogen, and high-sensitive C-reactive protein (hs-CRP). Dyslipidaemia was defined as LDL-cholesterol >100 mg/dl, HDL-cholesterol was lower than 40 or 50 mg/dl in males and females, respectively, triacylglycerol >150 mg/dl, and/or treatment with lipid-lowering agents. A blood aliquot was stored for DNA extraction and genetic screening.

2.2 | Laboratory measurements

HbA1c was measured by high-performance liquid chromatography using Diabetes Control and Complications Trial-aligned methods. Total cholesterol, high-density lipoprotein cholesterol, and triacylglycerol were determined by colourimetric enzymatic methods; low-density lipoprotein cholesterol was calculated by the Friedewald formula. Standard clinical laboratory methods have been employed for the measurement of glucose, serum creatinine, ALT, AST, GGT, uric acid, and fibrinogen. Measurement of serum hs-CRP was performed by using a multiplex detection 4-plex kit (Bio-Rad). Urinary albumin-to-creatinine ratio (ACR) was determined in at least three first-voided urine samples obtained with at least 1-month intervals in the year preceding the recruitment. All urine samples with abnormal sediments were discarded. Albumin (BNII; Dade Behring Diagnostic; intra- and inter-assay variation <2.0% and <3.5%, respectively) and creatinine (modified Jaffé reaction) were assayed on the same morning of collection.

Based on the geometric mean of three ACR values, the following categories were defined: normo-albuminuria (A1, <30 mg/g or <3.4 mg/mmol), micro-albuminuria or 'moderately increased albuminuria' (A2, 30–299 mg/g or 3.4–34.0 mg/mmol) and macro-albuminuria or 'severely increased albuminuria' (A3, ≥300 mg/g or ≥34 mg/mmol). Estimated glomerular filtration rate (eGFR) was calculated by the Chronic Kidney Disease Epidemiology Collaboration equation[27] and the following strata were defined: G1—eGFR ≥ 90; G2—eGFR 60–89; and ≥G3—eGFR < 60 ml/min/1.73 m^2. Renal impairment, be it albuminuria (ACR ≥30 mg/g or ≥3.4 mg/mmol), reduced estimated glomerular filtration rate (eGFR <60 ml/min/1.73 m^2) or both, defined the presence of diabetic kidney disease (DKD).

DNA was extracted from whole peripheral blood using an Archive Pure DNA blood kit (Eppendorf S.r.l.) according to the manufacturer's instructions and DNA samples were stored at −80°C until use. After exclusion of a few subjects with incomplete data (see below), samples for DNA extraction were available for 905 individuals (94.2% of the whole cohort; Figure S1) and they have been used to genotype for the tagging rs7896005 SNP located in the SIRT1 gene by means of ready-to-use TaqMan SNP assay (Life Technologies) on CFX Connect Real-Time System (Bio-Rad Laboratories). To confirm the accuracy of the genotyping results, 100 (about 10%) randomly selected samples were genotyped again with the same method, and no inconsistencies were observed.

2.3 | Assessment of diabetic complications

Diabetic retinopathy was assessed by retinal photography based on the photos of two fields per eye (disc-macula-temporal and disc-nasal) taken using a wide-angle (45°) mydriatic camera. Fundus features of the worse eye or retinal disease condition, including previous photocoagulation or surgical treatment, were used for staging according to the following categories: absent, mild, moderate, or severe non-proliferative, proliferative diabetic retinopathy, or maculopathy based on the Global Diabetic Retinopathy Project Group criteria.[28] For statistical analysis, patients with non-proliferative retinopathy of mild or moderate degree were classified as non-advanced diabetic retinopathy, whereas those with severe non-proliferative, proliferative, maculopathy, or blindness were grouped into the advanced, sight-threatening diabetic retinopathy category.

Diabetic peripheral neuropathy was assessed by means of the Michigan Neuropathy Screening Instrument Questionnaire,[29] presence of feet neuropathic ulcerations, assessment of knee and ankle reflexes, and measurement of vibration perception threshold using a bio-thesiometer applied bilaterally at the medial malleolus and the tip of the big toe.[30]

Presence of CV disease was ascertained on the basis of medical history of any major acute CV events, that is, myocardial infarction (MI), stroke, ischaemic foot ulcer or gangrene, amputation and coronary, carotid, and/or lower limb revascularisation. Any coronary heart disease (CHD) was defined as MI, stable and unstable angina, coronary revascularisation, or findings from coronary angiogram or coronary computed tomography angiogram or a 12-lead resting electrocardiogram (ECG) recorded in each subject and coded according to the Minnesota Code.[31] Cerebrovascular events have been defined as stroke or carotid revascularisation, while peripheral vascular disease was defined on a positive history of ischaemic ulceration, gangrene, amputation or lower limb revascularisation, or diagnosed on the presence of reduced or absent femoral and/or foot pulses and reduced ankle/brachial pressure ratio (<0.9).

2.4 | Assessment of outcomes in the prospective observation

All participants have been included in an observational study on the association between CV risk and presence of CV and microvascular complications. Thereafter, the cohort entered a prospective recording of incident major vascular events and all-cause mortality.

The primary outcome was the time of the first major CV event as defined above. Follow-up data for each patient and all-cause mortality were censored on 31 December 2017. The secondary outcomes were the time for the first coronary event as defined above. Secondary outcomes also included end stage renal disease (ESRD) and all-cause mortality.

Data on vascular outcomes (Figure S1) were available in 947 participants (98.5%) and were obtained, upon data anonymisation, in collaboration with the Regional Health Agency of the Tuscany Region (ARS Toscana) through hospital discharge registers. International Classification of Diseases, ninth Edition, Clinical Modification (ICD-9-CM) codes were used to detect vascular outcomes (Table S1). Follow-up was calculated for each single outcome. All events occurring between the date of enrolment and the end of follow-up or death were considered as incident.

Vital status was available for all participants and was verified over a mean follow-up of 13.1 ± 2.8 years (median 14.1, IQR 13.8–14.5) by interrogation of the Italian Health Card Database (http://sistemats1.sanita.finanze.it/wps/portal/).

2.5 | Statistical analyses

Genotype frequencies were preliminary tested for Hardy–Weinberg Equilibrium (HWE) by Pearson's chi-square analysis. Data are expressed as median (interquartile range [IQR]) and/or mean ± SD for continuous variables and number of cases and percentage for categorical variables. Continuous variables were compared by the Student's *t*-test or one-way analysis of variance (Welch robust test for equality of means when appropriate based on the Levene statistic) for normally distributed variables. Wilcoxon Sum-of-Ranks (Mann–Whitney) *U* test or Kruskal–Wallis test was used for variables with skewed distribution. Pearson χ^2 or the Fisher exact tests were applied to categories. For post hoc comparisons, Scheffe's test or Tamhane's test, Mann–Whitney *U* test, and χ^2 tests were used for normally distributed, not normally distributed, and categorical variables, respectively. Binary logistic regression analyses (including all variables of interest) were applied to assess the independent association of genotypes in the presence of CV events at baseline independent of several continuous and categorical variables including treatments and microvascular complications. Results of these analyses are expressed as OR and 95% confidence interval (CI).

A post-hoc power calculation has been performed to evaluate the statistical power of our sample size for the primary outcome, that is, major cardiovascular disease (CVD). Compared to the reference group (AA/AG subjects), our GG participants have a post-hoc power of 65% to detect the observed difference in the incidence of major CVD with an alpha error level of 5%.

Incidence of outcomes and crude mortality rates were described as events per 1000 person-years (PYs), with 95% exact Poisson Confidence Intervals (CI), as well after adjustment by age. Time to all-cause death or to each first outcome was plotted according to the genotypes as Kaplan-Meier (K-M) curves, with comparisons made using the log-rank test; risk estimates have been calculated by unadjusted Cox regression. For those outcomes for which the univariate Cox regression gave statistically significant estimates, multivariate Cox proportional hazard models were used to identify the independent effect of rs7896005 variants from key covariates such as sex, age, and diabetes exposure (Model 1), or as for Model 1 plus traditional and non-traditional CV risk factors (Model 2), and as for Model 2 plus presence of complications at baseline including prior CVD (Model 3). Results are expressed as HR and 95% CI. Analyses were performed also in prespecified subgroups according to the subjects' baseline demographics, medical histories, background habits, and baseline measurements. For these subgroup analyses, a multiplicative interaction term for each subgroup with the genotypes was added to the more comprehensive Cox model (Model 3).

A two-sided *p* value ≤0.05 was considered statistically significant. All statistical analyses were performed using SPSS package 25.0 version (IBM SPSS).

3 | RESULTS

We have evaluated 905 T2DM subjects (530 males, 58.6% and 375 females, 41.4%) with a mean age of 59.7 ± 7.2 years (median 61 years, IQR 56–65) and a mean duration of diabetes of 10.0 ± 8.6 years (median 8 years, IQR 3–15). The genotype distribution (AA, *n.* 82, 9.1%; AG, *n.* 391, 43.2%; GG, *n.* 432, 47.7%) met the Hardy–Weinberg equilibrium ($p = 0.890$), with a minor allele frequency of 0.3066.

A positive family history for T2DM and CVD was recorded in 520 (57.5%) and 446 (49.3%) individuals, respectively. The baseline clinical features of the cohort as a whole and stratified by genotypes are shown in Table 1. Major CV risk factors, mean HbA1c and HbA1c stratification, inflammatory biomarkers, and prevalence of microvascular complications were evenly distributed across genotypes, with the only exception of peripheral neuropathy that was more frequent in the GG genotype (26.4% vs. 19.9% in AA + AG; $p = 0.020$). No difference was apparent with respect to ongoing treatments.

3.1 | Cross-sectional evaluation: Relationship between rs7896005 and outcomes at baseline

Table 2 shows the prevalence of vascular outcome at baseline in the whole cohort and in the genotype groups. Prevalence of major CVD (*n.* 113, 12.5%, *p* for linear trend = 0.011), MI (*n.* 51, 5.6%, $p = 0.012$), any CHD (*n.* 134, 14.8%, $p = 0.006$) and peripheral artery disease (PAD; *n.* 126, 13.9%, $p = 0.007$) were higher in GG versus other genotypes. This difference was also confirmed by comparing GG versus pooled AA and AG subjects (Table 2). On the contrary, there was no association between genotypes and coronary revascularisation (*n.* 48, 5.3%) and cerebrovascular events (*n.* 33, 3.6%). By logistic regression analyses (Table S2), GG remained an independent

TABLE 1 Clinical characteristics of subjects as a whole and stratified by genotypes

	All subjects	AA genotype	AG genotype	GG genotype	*p value*
n. (%)	905	82 (9.1)	391 (43.2)	432 (47.7)	
Gender, M/F, *n* (%)	530/375 (58.6/41.4)	53/29 (64.6/35.4)	219/172 (56.0/44.0)	258/174 (59.7/40.3)	*0.282*
Age, years	59.7 ± 7.2	60.2 ± 6.9	59.7 ± 7.2	59.6 ± 7.2	*0.760*
Age at diagnosis, years	49.7 ± 9.7	51.5 ± 9.4	49.5 ± 10.2	49.5 ± 9.2	*0.197*
Diabetes duration, years	10.0 ± 8.6	8.7 ± 8.7	10.3 ± 9.0	10.1 ± 8.1	*0.322*
Positive family history for diabetes, *n* (%)	520 (57.5)	41 (50.0)	221 (56.5)	258 (59.7)	*0.233*
Positive family history for CVD, *n* (%)	446 (49.3)	31 (37.8)	196 (50.1)	219 (50.7)	*0.092*
BMI, kg/m^2	29.6 ± 5.2	29.9 ± 5.5	29.5 ± 5.2	29.7 ± 5.2	*0.739*
Waist circumference, cm	105.1 ± 1.9	106.2 ± 11.9	104.6 ± 11.5	105.3 ± 12.3	*0.467*
Active smokers, *n* (%)	189 (20.9)	19 (23.2)	87 (22.3)	83 (19.2)	*0.489*
Systolic BP, mmHg	143 ± 19	145 ± 20	142 ± 20	144 ± 19	*0.333*
Diastolic BP, mmHg	82 ± 10	82 ± 9	82 ± 10	83 ± 10	*0.311*
Fasting glucose, mg/dl	159 ± 44	154 ± 43	162 ± 46	157 ± 42	*0.236*
HbA1c, %	7.57 ± 1.20	7.48 ± 1.16	7.57 ± 1.20	7.58 ± 1.20	*0.770*
HbA1c strata, ≤7.0%, 7.1%–9.0%, >9.0%; *n* (%)	294/505/106 (32.5/55.8/11.7)	29/44/9 (35.4/53.6/11.0)	131/214/46 (33.5/54,7/11.8)	134/247/51 (31.0/57.2/11.8)	*0.916*
Total cholesterol, mg/dl	202 ± 37	202 ± 44	203 ± 35	201 ± 40	*0.912*
LDL cholesterol, mg/dl	130 ± 31	135 ± 35	131 ± 30	129 ± 32	*0.390*
HDL cholesterol, mg/dl	48 (41–57)	47 (42–53)	49 (42–58)	49 (40–57)	*0.338*
Triacylglycerol, mg/dl	133 (97–199)	142 (88–208)	130 (98–185)	137 (98–208)	*0.559*
ALT, U/L	21.8 ± 13.2	21.7 ± 9.2	21.8 ± 14.6	5.36 ± 1.41	*0.998*
AST, U/L	28.9 ± 24.2	31.1 ± 31.0	28.8 ± 25.6	28.6 ± 21.3	*0.693*
GGT, U/L	40.0 ± 80.0	42.3 ± 43.0	38.9 ± 61.9	40.6 ± 101.6	*0.925*
Fibrinogen, mg/dl	360 ± 81	354 ± 79	361 ± 80	360 ± 81	*0.768*
hs-CRP, mg/L	4.26 ± 7.38	3.71 ± 4.27	4.35 ± 7.89	4.29 ± 7.38	*0.772*
Serum creatinine, mg/dl	0.91 ± 0.52	0.88 ± 0.21	0.93 ± 0.71	0.89 ± 0.32	*0.560*
eGFR (CKD-EPI), ml/min/1.73 m^2	85.8 ± 16.7	86.3 ± 15.2	85.6 ± 17.3	85.9 ± 16.5	*0.911*
eGFR strata, G1 ≥90, G2 60–90, G3 <60 ml/min/1.73 m^2, *n* (%)	447/396/62 (49.4/43.8/6.9)	44/32/6 (53.7/39.0/7.3)	194/167/30 (49.6/42.7/7.7)	209/197/26 (48.4/45.6/6.0)	*0.718*
Uric acid, mg/dl	5.34 ± 1.48	5.40 ± 1.67	5.30 ± 1.52	5.36 ± 1.41	*0.805*
UACR, mg/g	6.12 (3.39–15.79)	6.19 (3.57–13.60)	6.08 (3.39–15.36)	6.12 (3.33–16.95)	*0.787*
UACR strata, A1 <30, A2 30–299, A3 ≥300 mg/g, *n* (%)	750/122/33 (82.9/13.5/3.6)	70/11/1 (85.4/13.4/1.2)	327/51/13 (83.6/13.0/3.3)	353/60/19 (81.7/13.9/4.4)	*0.668*
DKD, *n* (%)	187 (20.7)	16 (19.5)	79 (20.2)	92 (21.3)	*0.895*
Retinopathy, *n* (%)					
Non-advanced	165 (18.2)	13 (15.9)	80 (20.5)	72 (16.7)	
Advanced	95 (10.5)	5 (6.1)	40 (10.2)	50 (11.6)	*0.323*

(Continues)

TABLE 1 (Continued)

	All subjects	AA genotype	AG genotype	GG genotype	*p value*
Peripheral neuropathy, *n* (%)	208 (23.0)	17 (20.7)	77 (19.7)	114 (26.4)	*0.065*
Hypertension,[a] *n* (%)	721 (79.7)	64 (78.0)	308 (78.8)	349 (80.8)	*0.719*
Dyslipidaemia,[b] *n* (%)	759 (83.9)	72 (87.8)	331 (84.7)	356 (82.4)	*0.407*
BP-lowering agents, *n* (%)	491 (54.3)	37 (45.1)	216 (55.2)	238 (55.1)	*0.220*
RAS-blockers, *n* (%)	392 (43.3)	29 (35.4)	173 (44.2)	190 (44.0)	*0.312*
Lipid-lowering drugs, *n* (%)	287 (31.7)	21 (25.6)	124 (31.7)	142 (32.9)	*0.432*
Anti-platelet drugs, *n* (%)	183 (20.2)	12 (14.6)	88 (22,5)	83 (19.2)	*0.210*
Metformin, *n* (%)	533 (58.9)	49 (59.8)	232 (59.3)	252 (58.3)	*0.945*
Secretagogues, *n* (%)	441 (48.7)	44 (53.7)	196 (50.1)	201 (46.5)	*0.379*
Thiazolidinediones, *n* (%)	46 (5.1)	7 (8.5)	21 (5.4)	18 (4.2)	*0.241*
Insulin, *n* (%)	230 (25.4%)	20 (24.4)	96 (24.6)	114 (26.4)	*0.813*

Note: Data are expressed as mean ± sd or as median and interquartile range (IQR) or as number and percentage.

[a]Hypertension was defined as systolic BP > 140 mmH or diastolic BP > 90 mmHg and/or treatment with BP-lowering agents.

[b]Dyslipidaemia was defined as low-density lipoprotein (LDL) cholesterol >100 mg/dl, high-density lipoprotein (HDL) cholesterol lower than 40 or 50 mg/dl (in males and females, respectively), triacylglycerol >150 mg/dl and or treatment with lipid-lowering agents.

TABLE 2 Prevalence of vascular events at baseline in the whole cohort, in subjects stratified by genotypes, and in GG versus AA and AG combined

	All subjects	AA	AG	GG	*p (p for linear association)*	AA/AG	*p*
n. (%)	905	82 (9.1)	391 (43.2)	432 (47.7)	--	473 (52.3)	--
Major CVD,[a] *n* (%)	113 (12.5)	5 (6.1)	43 (11.0)	65 (15.0)	*0.040 (0.011)*	48 (10.1)	*0.026*
Myocardial infarction, *n* (%)	51 (5.6)	1 (1.2)	18 (4.6)	32 (7.4)	*0.042 (0.012)*	19 (4.0)	*0.027*
Any CHD,[b] *n* (%)	134 (14.8)	5 (6.1)	53 (13.6)	76 (17.6)	*0.018 (0.006)*	58 (12.3)	*0.024*
Coronary revascularisation, *n* (%)	48 (5.3)	4 (4.9)	19 (4.9)	25 (5.8)	*0.825 (0.577)*	23 (4.9)	*0.535*
Cerebrovascular events,[c] *n* (%)	33 (3.6)	5 (6.1%)	14 (3.6)	14 (3.2)	*0.447 (0.303)*	19 (4.0)	*0.534*
Peripheral artery disease,[d] *n* (%)	126 (13.9)	6 (7.3)	47 (12.0)	73 (16.9)	*0.025 (0.007)*	53 (11.2)	*0.013*

[a]Major CVD = MI, stroke, ischaemic foot ulcer or gangrene, amputation and coronary, carotid, and/or lower limb revascularisation.

[b]Any CHD = MI, stable and unstable angina, coronary revascularisation or findings from coronary angiogram or coronary computed tomography angiogram or a 12-lead resting ECG recorded in each subject and coded according to the Minnesota Code (31).

[c]Cerebrovascular events = stroke or carotid revascularisation.

[d]Peripheral vascular disease = positive history of ischaemic ulceration, gangrene, amputation or lower limb revascularisation, or diagnosed on the presence of reduced or absent femoral and/or foot pulses and reduced ankle/brachial pressure ratio (<0.9).

covariate of major CVD (OR 1.628, 95% CI 1.074–2.467, $p = 0.022$) with independent effects for age, male gender, and retinopathy (both non-advanced and advanced), and marginal effects for hypertension, dyslipidaemia, and peripheral neuropathy. Also, GG remained an independent covariate of MI (OR 2.034. 95% CI 1.099–3.763, $p = 0.024$, with additional effects for male gender and hypertension, and marginal effect for dyslipidaemia; Table S2), any CHD (OR 1.691, 95% CI 1.135–2.519, $p = 0.010$) with additional effects for male gender, age and diabetes duration (DD), BMI, hypertension, dyslipidaemia, ACR (Table S3), and PAD (OR 1.679, 95% CI 1.103–2.556, $p = 0.016$), with additional effects for male gender, age, active smoking, peripheral neuropathy and retinopathy, mainly advanced retinopathy (Table S3).

3.2 | The prospective observational study: Relationship between rs7896005 and outcomes over the follow-up

Table 3 shows the incidence of vascular outcomes and all cause-mortality according to genotypes. A total of 258 major CV events (28.5%; incidence density 25.39 × 1000 person-years [PYs]; 95% CI

TABLE 3 Incidence of outcomes and all-cause mortality rates according to genotypes, unadjusted, and age adjusted

	Events	% events	Events per 1000 patient-years (95% CI) unadjusted	*Poisson regression model, p*	Events per 1000 patient-years (95% CI) age-adjusted	*Poisson regression model, p*
Major CVD						
AA/AG	119	25.2	21.79 (18.20–26.07)		21.04 (17.52–25.26)	
GG	139	32.2	29.57 (25.04–34.92)	<0.0001	28.72 (24.24–34.02)	<0.0001
	K-M, log rank 5.985; $p = 0.014$					
CHD events						
AA/AG	73	15.4	12.72 (10.11–15.99)		12.42 (9.85–15.66)	
GG	96	22.2	19.45 (15.92–23.75)	<0.0001	19.17 (15.66–23.46)	<0.0001
	K-M, log rank 7.511; $p = 0.006$					
Hospitalisation for heart failure						
AA/AG	33	7.0	5.42 (3.86–7.63)		4.70 (3.27–6.76)	
GG	46	11.6	8.45 (6.33–11.28)	<0.0001	7.35 (5.36–10.07)	<0.0001
	K-M, log rank 4.018; $p = 0.045$					
PAD events						
AA/AG	11	2.3	1.78 (0.98–3.22)		1.64 (0.89–3.00)	
GG	25	5.8	4.54 (3.07–6.72)	<0.0001	4.69 (2.77–6.36)	<0.0001
	K-M, log rank 7.261; $p = 0.007$					
All-cause death						
AA/AG	104	22.0	16.75 (13.82–20.29)		14.58 (11.86–17.93)	
GG	114	26.4	20.31 (16.90–24.40)	0.156	18.84 (14.63–21.75)	0.140
	K-M, log rank 2.216; $p = 0.137$					

Abbreviations: CI, confidence interval; K-M, Kaplan-Meier.

22.47–28.68) occurred over a mean follow-up of 11.2 ± 4.4 years (median 13.9, IQR 8.4–14.3 years); incidence rates were 25.2% in AA/AG (119 out of 473, 21.79 × 1000 PYs; reference) and 32.2% in GG (139 out of 432, 29.57 × 1000 PYs; K-M, log rank = 5.985, $p = 0.014$; unadjusted HR 1.356, 95% CI 1.061–1.732, $p = 0.015$; Figure 1A).

Coronary events occurred in 169 out of 905 subjects (18.7%; incidence density 15.83 × 1000 PYs; 95% CI 13.61–18.40) over a mean follow-up of 11.8 ± 4.1 years (median 13.9, IQR 9.8–14.3 years); incidence was 15.4% in AA/AG (73 out of 473, 12.72 × 1000 PYs, reference) and 22.2% in GG (96 out of 432, 19.45 × 1000 PYs; log rank 7.511, $p = 0.006$; unadjusted HR 1.526, 95% CI 1.125–2.069, $p = 0.007$; Figure 1B).

Hospitalisations for heart failure (HF) occurred in 79 participants (8.7%; incidence density 6.85 × 1000 PYs; 95% CI 5.50–8.54) over a mean follow-up of 12.7 ± 3.1 years (median 14.1, IQR 13.1–14.4 years); incidences were 7.0% in AA/AG (33 out of 473, 5.42 × 1000 PYs) and 11.6% in GG (46 out of 432, 8.45 × 1000 PYs; log rank 4.018, $p = 0.045$; unadjusted HR 1.574, 95% CI 1.006–2.461, $p = 0.047$; Figure 1C).

Peripheral artery disease events occurred in 36 participants (4.0%; incidence density 3.08 × 1000 PYs; 95% CI 2.22–4.28) over a mean follow-up of 12.9 ± 3.0 years (median 14.1, IQR 13.7–14.5 years); incidence rates were 2.3% in AA/AG (11 out of 473, 1.78 × 1000 PYs) and 5.8% in GG (25 out of 432, 4.54 × 1000 PYs; log rank 7.261, $p = 0.007$; unadjusted HR 2.560, 95% CI 1.260–5.203, $p = 0.009$; Figure 2A).

No association was observed with cerebrovascular events ($n = 96$; 10.6%) or ESRD ($n = 69$, 7.6%; Figure 2B,C). All-cause death

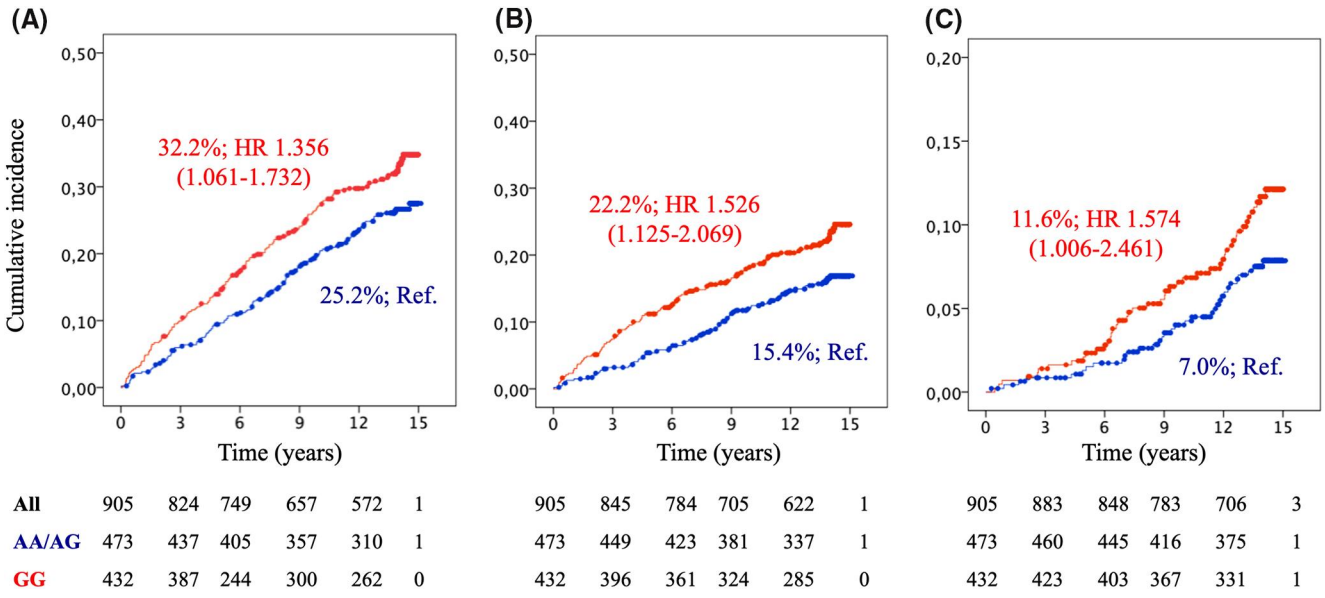

FIGURE 1 Kaplan-Meier (K-M) curves describing the cumulative incidences of major vascular events in subjects stratified by the rs7896005 variant (GG, red line vs. AA/AG, blue line). Percentages of events and Cox proportional unadjusted hazard ratios (HRs, 95% CI) are shown for each group. Panel (A): major cardiovascular (CV) events; panel (B): coronary heart disease (CHD) events; panel (C): hospitalisations for heart failure (HF)

occurred in 24.1% (n = 218) over a mean follow-up of 13.1 ± 2.8 years (median 14.1, IQR 13.8–14.5) with an incidence density of 18.44 × 1000 PYs; 95% CI 16.14–21.05 with no differences in death rate in AA/AG versus GG (Figure S2).

In all-adjusted Cox regression models, as compared to AA/AG, the GG genotype remained an independent risk factor for major CV events, coronary artery diseases, hospitalisation for HF, and PAD (Tables 4 and 5). Even with the more stringent model (Model 3), GG was associated with incidence of major CV events (HR 1.296; 95% CI 1.007–1.668, p = 0.044), with an independent effect for age, male sex, DD, active smoking, dyslipidaemia, peripheral neuropathy, advanced retinopathy and, in particular, prior CVD (Table 4). Consistently, GG was associated with incident coronary artery disease (1.522; 1.113–2.080, p = 0.008) with independent effects for male sex, DD, HbA1c, active smoking, dyslipidaemia, retinopathy, prior CVD and, inversely, eGFR (Table 4); hospitalisation for HF (1.457; 0.919–2.309, p = 0.109) and PAD (2.225; 1.057–4.684, p = 0.035; Table 5). The results were similar by using urine albumin-to-creatinine ratio (UACR) and eGFR as continuous variables or as categories or strata (data not shown).

There were no significant interactions between all prespecified subgroups based on demographics (sex, age, or BMI stratified by median value), medical history (prior CVD), background habits (active smoking), or baseline HbA1c stratified by median value and the effect of the GG genotype (Table S4). Nevertheless, in individuals with HbA1c >7.45% (median value) and in those with greater BMI, GG genotype was associated with higher hazard ratios (HRs) for each one of the vascular outcomes. Similarly, GG had higher HRs for major CVD (p for interaction = 0.060), CHD events, and PAD in subjects with prior CV disease at baseline (Table S4).

4 | DISCUSSION

In a cohort of T2DM individuals, the rs7896005 GG genotype of the *SIRT1* gene was independently associated with a composite of major CV diseases (CVD) both in a cross-sectional as well as in a prospective analysis. In particular, the rs7896005 GG was associated with major CVD, CHD events, hospitalisation for HF, and PAD events independent of multiple covariates and confounding factors, whereas no association was found with the incidence of cerebrovascular events, ESRD, and all-cause mortality.

In the Rotterdam Study, *SIRT1* genetic haplotypes were not associated with mortality in the overall population, although all-cause mortality increased by 50% (95% CI 1.1–2.2) in T2DM subjects.[26] We were not able to replicate that finding, but data on the association between *SIRT1* variant and life expectancy are quite inconsistent. In the Leiden 85-plus Study, *SIRT1* sequence variations did not affect ageing in the German, Dutch, and Belgian populations.[14,15] Consistently, *SIRT1* SNPs showed no association with mortality in the Concord Health and Ageing in Men Project Australian study.[17] On the contrary, the minor A allele of the *SIRT1* SNP rs7896005 was significantly associated with longevity in two small cohorts of Caucasian subjects,[12] while carriers of the minor C allele of the rs12778366 SNP had a reduced risk of death in a population-based cohort of white individuals of Dutch descent.[13] Finally, two studies performed in different Chinese populations reported a potential effect of SIRT1 gene variants on ageing in one case,[11] and no association in the other.[16] While there are no simple reasons to justify such wide differences, large ethnic variations in allele frequencies might be the most obvious explanation as the frequency of the A allele of rs7896005 has been found to range from less than 0.30 to >0.80.[12]

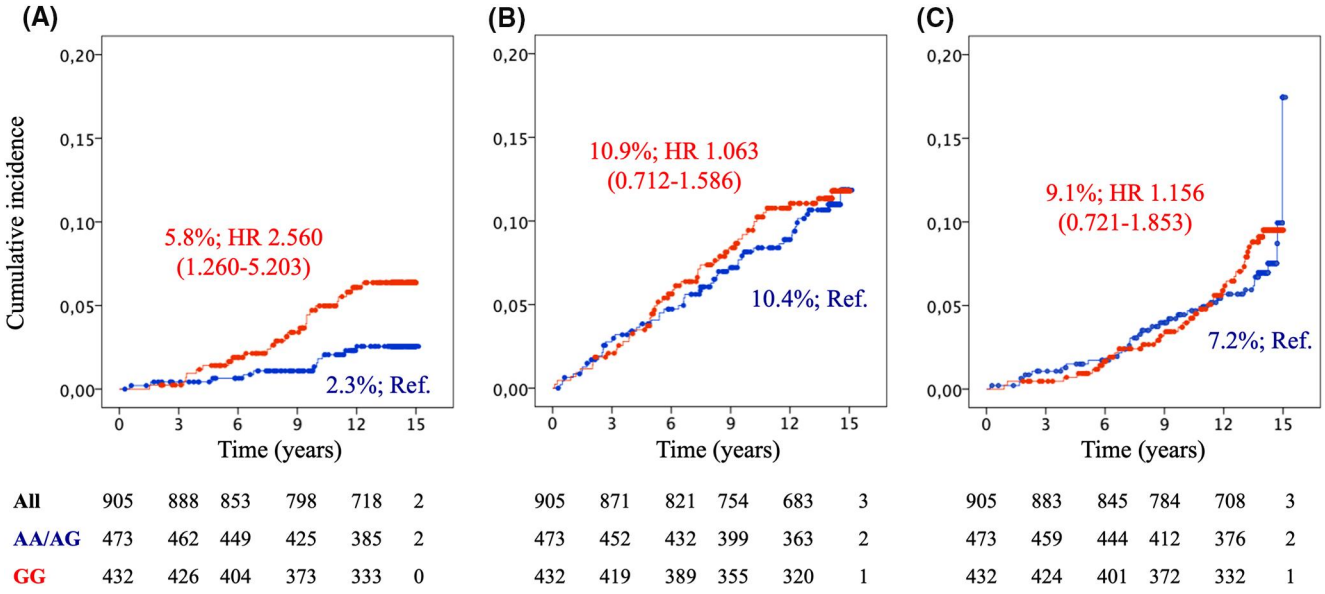

FIGURE 2 Kaplan-Meier (K-M) curves describing the cumulative incidences of major vascular events in subjects stratified by the rs7896005 variant (GG, red line vs. AA/AG, blue line). Percentages of events and Cox proportional unadjusted hazard ratio (HR) (HRs, 95% CI) are shown for each group. Panel (A): peripheral artery disease (PAD) events; panel (B): cerebrovascular events; panel (C): end stage renal disease (ESRD)

Even more scanty are the data on *SIRT1* gene variants and CVD, and genetic analyses from a large database (genome-wide association study) about the role of *SIRT1* SNPs are still pending.[18] To the best of our knowledge, this is the first study to explore such an association both in a cross-sectional and in a prospective manner in the same cohort of T2DM subjects. In both analyses, subjects homozygous for the G allele have greater risk for several CV outcomes as compared to those carrying the A allele. In the cross-sectional study, GG subjects had an odds ratio of 2.034 for the risk of MI with respect to AA/AG individuals. Moreover, upon 'fully' adjusted logistic regressions, the GG genotype was independently associated with a 60–70% increase in the prevalence of major CVD, any CHD, and PAD (Tables S2 and S3).

During an average 13-year follow-up, the GG genotype was found to confer greater susceptibility to major CVD, CHD, and PAD events as well as the risk of hospitalisation for HF. In the more comprehensive model of Cox regression (Model 3) the estimates of risk (HRs) ranged from an increase of about 30% in the incidence of major CVD to more than doubling of the incidence of PAD. These estimates were independent of a large set of confounders including demographic factors and exposure to diabetes (Model 1), the same factors plus other baseline conventional and emerging CV risk factors (Model 2), and, finally, even after further adjustment for coexistence of diabetic complications including prior major CVD (Model 3). In this regard, it is of interest to observe that the strength of the risk estimates, that is, the numerical entity of the HRs, for each vascular outcome was only slightly attenuated moving from unadjusted Cox regression analysis (Figures 1 and 2) to increasingly complex regression models (Tables 4 and 5). The independent effect of the GG haplotype is further supported by the observation that, at baseline, the different genetic subgroups were superimposable with respect to several clinical features, metabolic parameters, microvascular complications, and treatments (Table 1).

Several experimental studies[18–20] may offer ground for our observation, while data in human are more fragmented. Kilic et al.[21] reported that the prevalence of mutant genotypes and alleles for the rs7069102 and rs2273773 *SIRT1* SNPs was higher in patients with coronary artery stenosis compared to the control group. In a case-control study, Cheng et al.[24] found that the G allele and the CG/GG genotypes of the tagSNP rs7069102 of *SIRT1* were associated with an increased risk of MI (OR 1.57 and 1.64, respectively) in a Chinese population; consistent results were obtained for a haplotype including three tagSNPs of *SIRT1* (OR 1.41). In a more recent study, a haplotype derived from three *SIRT1* SNPs alleles was found to exert a protective effect on the risk of coronary artery disease.[25] Other studies showed that some *SIRT1* SNPs were associated with coronary artery calcification[32] and with carotid atherosclerosis.[23] In the latter study, upon adjustment for CV risk factors, genetic polymorphisms at *SIRT1* were found to be associated with common carotid intima-media thickness with a greater effect in women.[23] Moreover, *SIRT1* expression was found to be lower in human atherosclerotic carotid arteries compared with non-diseased arteries.[18] We did not have the opportunity to assess carotid arteries in our study, but it may be worth highlighting that we found no association between the rs7896005 SNP and cerebrovascular events (Table 2 and Figure 2B).

While an association between the GG haplotype and main CV events was found, no association was apparent for incident ESRD. This finding fits with the lack of an association between the rs7896005 genotypes and UACR and eGFR levels, UACR categories and eGFR strata, or DKD phenotypes at baseline in spite of a plausible role of Sirt1 in the kidney. Sirt1 is indeed expressed in all renal structures and a reduction of its expression has been observed in the

TABLE 4 Adjusted Cox regression analyses assessing the role of rs7896005 GG genotype as an independent covariate of the incidence of major cardiovascular disease (CVD; *n.* 258, 28.5%) and of the incidence of coronary artery disease (*n.* 169, 18.7%) in the prospective observation

	Major CVD			Coronary artery disease		
	HR	95% CI	*p*	HR	95% CI	*p*
Model 1						
Age, years	1.044	1.025–1.064	<0.0001	1.034	1.011–1.059	0.004
Gender, males	2.324	1.764–3.063	<0.0001	2.056	1.469–2.876	<0.0001
Diabetes duration, years	1.020	1.006–1.034	0.005	1.013	0.995–1.031	0.150
HbA1c, %	1.123	1.011–1.248	0.030	1.165	1.026–1.324	0.019
rs7896005, GG	1.362	1.066–1.741	0.013	1.539	1.135–2.087	0.006
Model 2						
Age, years	1.044	1.024–1.065	<0.0001	1.034	1.009–1.059	0.008
Gender, males	2.458	1.831–3.300	<0.0001	2.125	1.485–3.041	<0.0001
Diabetes duration, years	1.023	1.009–1.037	0.001	1.016	0.998–1.035	0.074
HbA1c, %	1.103	0.989–1.230	0.078	1.144	1.003–1.306	0.046
BMI, kg/m^2	1.010	0.983–1.039	0.467	1.002	0.968–1.037	0.916
Active smoking	1.480	1.101–1.990	0.009	1.423	0.989–2.049	0.058
Hypertension[a]	1.326	0.930–1.890	0.119	1.217	0.792–1.868	0.370
Dyslipidaemia[b]	1.763	1.191–2.610	0.005	2.092	1.240–3.530	0.006
Uric acid, mg/dl	0.989	0.904–1.082	0.810	1.012	0.904–1.133	0.835
hs-CRP, mg/L	1.015	1.000–1.030	0.050	1.018	1.001–1.035	0.041
rs7896005, GG	1.374	1.074–1.757	0.011	1.556	1.146–2.113	0.005
Model 3						
Age, years	1.023	1.001–1.046	0.041	1.013	0.986–1.040	0.348
Gender, males	2.155	1.602–2.899	<0.0001	1.933	1.346–2.777	<0.0001
Diabetes duration, years	1.019	1.004–1.035	0.015	1.026	1.006–1.046	0.011
HbA1c, %	1.088	0.972–1.217	0.144	1.179	1.032–1.348	0.011
BMI, kg/m^2	1.000	0.972–1.028	0.973	1.001	0.967–1.035	0.977
Active smoking	1.457	1.081–1.962	0.013	1.469	1.017–2.124	0.041
Hypertension[a]	1.195	0.833–1.714	0.333	1.213	0.785–1.874	0.354
Dyslipidaemia[b]	1.704	1.147–2.533	0.008	1.974	1.132–3.238	0.015
Uric acid, mg/dl	0.955	0.863–1.056	0.369	0.985	0.872–1.113	0.807
hs-CRP, mg/L	1.014	0.999–1.030	0.074	1.017	1.000–1.035	0.056
ACR ratio, mg/g	1.000	0.999–1.000	0.862	1.000	1.000–1.001	0.202
eGFR CKD-EPI, ml/min/1.73 m^2	0.993	0.984–1.003	0.179	0.990	0.978–1.001	0.080
Peripheral neuropathy	1.673	1.250–2.240	0.001	1.255	0.863–1.825	0.234
Retinopathy			0.113			<0.0001
Non-advanced	0.864	0.611–1.221	0.407	0.588	0.377–0.918	0.019
Advanced	0.627	0.404–0.973	0.037	0.299	0.157–0.568	<0.0001
Prior major CVD	3.149	2.339–4.241	<0.0001	2.959	2.055–4.262	<0.0001
rs7896005, GG	1.296	1.007–1.668	0.044	1.522	1.113–2.080	0.008

[a]Hypertension was defined as systolic BP > 140 mmHg or diastolic BP > 90 mmHg and/or treatment with BP-lowering agents.

[b]Dyslipidaemia was defined as LDL cholesterol >100 mg/dl, HDL cholesterol lower than 40 or 50 mg/dl (in males and females, respectively), triacylglycerol >150 mg/dl and or treatment with lipid-lowering agents.

TABLE 5 Adjusted Cox regression analyses assessing the role of rs7896005 GG genotype as an independent covariate of the incidence of hospitalisation for heart failure (HF) (*n.* 79, 8.7%) and incidence of peripheral artery disease (PAD) (*n.* 36, 4.0%) in the prospective observation

	Hospitalisation for heart failure			Peripheral artery disease		
	HR	95% CI	*p*	HR	95% CI	*p*
Model 1						
Age, years	1.080	1.039–1.122	<0.0001	1.058	1.004–1.116	0.035
Gender, males	1.009	0.643–1.582	0.970	3.878	1.677–8.967	0.002
Diabetes duration, years	1.033	1.009–1.057	0.007	1.032	0.996–1.069	0.082
HbA1c, %	1.156	0.963–1.388	0.120	1.481	1.155–1.899	0.002
rs7896005, GG	1.546	0.988–2.419	0.056	2.561	1.258–5.214	0.010
Model 2						
Age, years	1.077	1.034–1.121	<0.0001	1.062	1.006–1.122	0.030
Gender, males	1.172	0.717–1.915	0.526	4.750	1.947–11.586	0.001
Diabetes duration, years	1.041	1.016–1.066	0.001	1.038	1.001–1.076	0.043
HbA1c, %	1.178	0.974–1.423	0.091	1.507	1.151–1.973	0.003
BMI, kg/m^2	1.068	1.022–1.115	0.003	1.067	0.995–1.144	0.069
Active smoking	1.074	0.558–2.070	0.830	2.506	1.144–5.490	0.022
Hypertension[a]	3.086	1.108–8.597	0.031	1.483	0.517–4.256	0.464
Dyslipidaemia[b]	1.351	0.667–2.736	0.403	2.239	0.656–7.646	0.198
Uric acid, mg/dl	1.150	0.981–1.349	0.086	1.052	0.820–1.349	0.690
hs-CRP, mg/L	1.000	0.967–1.034	0.998	1.022	0.992–1.053	0.150
rs7896005, GG	1.547	0.988–2.423	0.056	2.774	1.358–5.667	0.005
Model 3						
Age, years	1.068	1.021–1.117	0.004	1.001	0.942–1.064	0.969
Gender, males	0.991	0.597–1.645	0.973	3.463	1.435–8.355	0.006
Diabetes duration, years	1.042	1.015–1.070	0.003	1.005	0.964–1.048	0.816
HbA1c, %	1.147	0.946–1.390	0.163	1.453	1.091–1.934	0.011
BMI, kg/m^2	1.061	1.016–1.109	0.008	1.049	0.979–1.125	0.176
Active smoking	1.035	0.533–2.009	0.919	2.141	0.962–4.764	0.062
Hypertension[a]	2.929	1.046–8.198	0.041	1.975	0.540–7.219	0.304
Dyslipidaemia[b]	1.228	0.598–2.523	0.576	1.148	0.387–3.408	0.804
Uric acid, mg/dl	1.150	0.957–1.381	0.135	0.882	0.658–1.182	0.401
hs-CRP, mg/L	1.001	0.967–1.036	0.960	1.024	0.991–1.059	0.160
ACR ratio, mg/g	1.001	1.000–1.002	0.001	0.999	0.997–1.001	0.284
eGFR CKD-EPI, ml/min/1.73 m^2	1.009	0.992–1.027	0.309	0.975	0.950–1.001	0.059
Peripheral neuropathy	1.686	1.018–2.792	0.042	2.571	1.188–5.564	0.017
Retinopathy			0.575			0.218
Non-advanced	0.754	0.407–1.369	0.369	2.095	0.885–4.963	0.093
Advanced	0.721	0.333–1.561	0.407	1.186	0.440–3.197	0.736
Prior major CVD	2.062	1.196–3.554	0.009	4.552	2.198–9.426	<0.0001
rs7896005, GG	1.457	0.919–2.309	0.109	2.225	1.057–4.684	0.035

[a]Hypertension was defined as systolic BP > 140 mmHg or diastolic BP > 90 mmHg and/or treatment with BP-lowering agents.

[b]Dyslipidaemia was defined as LDL cholesterol >100 mg/dl, HDL cholesterol lower than 40 or 50 mg/dl (in males and females, respectively), triacylglycerol >150 mg/dl and or treatment with lipid-lowering agents.

glomeruli and the tubulointerstitial compartments of subjects with mild to severe DKD.[33–35] A possible effect of ethnicity-related factors could be considered as well since *SIRT1* SNPs variants have been claimed to predispose towards DKD in Japanese[36] and Chinese[37] populations.

No significant interactions were observed across prespecified subgroups with respect to the effect of the GG genotype although some suggestions may emerge that may be worth keeping in mind for future. Thus, although the size of our cohort makes results of subgroup analyses underpowered, a signal for greater effects of the GG genotype seems to be there for subjects with higher BMI or with worse glycaemic control (HbA1c) at baseline. A potential interaction between these features and the effects of the *SIRT1* genetic variants is plausible as previously suggested.[26] Thus, previous studies have reported an association of *SIRT1* gene variants with BMI and risk of obesity[38] and with visceral obesity and its severity.[39] Obesity[40] and poor glycaemic control[41] are often associated with worse CV risk profile and increased oxidative stress and *SIRT1* have been suggested to exert a protective effect against the latter.[42] Another potential interaction that may deserve future attention is the one with existing CV disease. Such a potential interaction is not surprizing as the negative effect of the GG genotype can be well expected to impact more on those with a prior CV event due to a much greater CV risk.

Finally, the association between *SIRT1* genetic variants and CV risk in our population may also rely on plausible pathogenetic mechanisms.[4–6,18] At the arterial level, Sirt1 is involved in the activation of endothelial nitric oxide synthase, in the inhibition of NF-kB activity in endothelial cells and macrophages,[18] in the protection of VSMCs against DNA damage and media degeneration.[18,19] Furthermore, it plays a role in the attenuation of inflammatory and oxidative injury,[19,20] in the control of apoptosis, autophagy and senescence of vessel cells,[19] in the stimulation of antioxidant defences[22] and in the maintenance of the vascular repair capacity of endothelial progenitor cells.[43] Thus, the entire atherogenic process can be to some extent under the control of the Sirt1.[44]

Higher plasma levels of SIRT1 have been reported in patients with stroke,[45,46] but whether SIRT1 activation improves stroke outcomes is still a matter of controversy.[47] SIRT1 is involved in several pathways of ischaemic cerebral protection[48] and its overexpression is claimed to play a key role during ischaemia/hypoxia by protecting against cellular stress, controlling the metabolic pathways, or further contributing to the injury.[48] In our study, we did not observe an association between the rs7896005 GG genotype and cerebrovascular outcome. The low number of events, however, may have precluded the possibility to fully explore a potential association although it is intriguing that associations between the SIRT1 genotype and events (hospitalisation for HF, peripheral artery diseases) also occurred at a similarly low rate. Finally, it is necessary to emphasise that we could not distinguish between ischaemic and haemorrhagic stroke.

In order to put our results in a more balance perspective, limitations and strengths of our study need to be taken into consideration. First, our study population included T2DM subjects recruited in a single hospital-based centre and, as such, may not represent the general population, although the genotype distribution was in the Hardy–Weinberg equilibrium. Nonetheless, only Caucasian subjects were recruited so that our findings need to be reproduced in larger samples and in different cohorts to allow extrapolation of our results to a broader population. Second, we could not retrieve reliable data about the cause of death, including CV death, from the Regional Discharge Registry, somewhat limiting the detail of study results. Furthermore, the rs7896005 is a non-coding tagSNP implying it must be linked with one or more functional variants within the *SIRT1* gene or its regulatory regions. To make this even more important is the fact that we have not determined the relationship between the SNP and Sirt1 expression, an association that has been reported by others.[21] Thus, no conclusion can be drawn in terms of the actual function and activity of Sirt1 under the present circumstances. On the other hand, our approach has the advantage to have assessed the same population in a cross-sectional as well as in a longitudinal manner thus lending strength to our results. Finally, of interest, significant associations of the *SIRT1* rs7896005 SNP with major CVD and other vascular outcomes have been observed despite a post-hoc power calculation of 65% for the primary outcome.

In summary, our study provides the first evidence that the G allele of the *SIRT1* rs7896005 tagSNP is associated with an increased risk of major CV diseases in Caucasian individuals with type 2 diabetes. These associations were independent of a wide set of confounding factors and have been first detected in a cross-sectional study and then confirmed in a long-term prospective design.

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CONFLICT OF INTEREST

The authors declare that they have no competing interest.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Ethics Committee of the University of Pisa approved the study protocol and written informed consent was obtained from all participants before any study procedure.

CONSENT FOR PUBLICATION

All authors consented for the publication of the manuscript.

AUTHOR CONTRIBUTIONS

Study conception: Angela Dardano, Daniela Lucchesi, Monia Garofolo and Giuseppe Penno; study design: Stefano Del Prato, Monia Garofolo and Giuseppe Penno; genetic testing: Daniela Lucchesi and Veronica Sancho Bornez; data collection: Elisa Gualdani, Paolo Francesconi and Pierpaolo Falcetta; data preparation and analysis:

Angela Dardano, Daniela Lucchesi, Monia Garofolo and Giuseppe Penno; first draft: Stefano Del Prato, Monia Garofolo and Giuseppe Penno; study critical revision and manuscript draft: all authors.

DATA AVAILABILITY STATEMENT

Data collected for this study can be shared and made available upon reasonable request to the corresponding author and subject to an approved proposal and data access agreement.

TRANSPARENT PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/dmrr.3523.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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