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Elevated plasma hepcidin concentrations are associated with an increased risk of mortality and nonfatal cardiovascular events in patients with type 2 diabetes: a prospective study

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Abstract

Background The effect of plasma hepcidin concentrations on the long-term risk of developing adverse cardiovascular outcomes in people with type 2 diabetes mellitus (T2DM) is unclear.

Methods We followed for a median of 55.6 months 213 outpatients with established T2DM (45.5% women, mean age 69 ± 10 years; BMI 28.7 ± 4.7 kg/m^2; median diabetes duration 11 years). Baseline plasma ferritin and hepcidin concentrations were measured with an electrochemiluminescence immunoassay and mass spectrometry-based assay, respectively. The primary study outcome was a composite of all-cause mortality or incident nonfatal cardiovascular events (inclusive of myocardial infarction, permanent atrial fibrillation, ischemic stroke, or new hospitalization for heart failure).

Results 42 patients developed the primary composite outcome over a median follow-up of 55.6 months. After stratifying patients by baseline hepcidin tertiles [1st tertile: median hepcidin 1.04 (IQR 0.50–1.95) nmol/L, 2nd tertile: 3.81 (IQR 3.01-4-42) nmol/L and 3rd tertile: 7.72 (IQR 6.37–10.4) nmol/L], the risk of developing the primary composite outcome in patients in the 3rd tertile was double that of patients in the 1st and 2nd tertile combined (unadjusted hazard ratio [HR] 2.32, 95%CI 1.27–4.26; $p = 0.007$). This risk was not attenuated after adjustment for age, sex, adiposity measures, smoking, hypertension, statin use, antiplatelet medication use, plasma hs-C-reactive protein and ferritin concentrations (adjusted HR 2.53, 95%CI 1.27–5.03; $p = 0.008$).

Conclusions In outpatients with T2DM, higher baseline hepcidin concentrations were strongly associated with an increased long-term risk of overall mortality or nonfatal cardiovascular events, even after adjustment for established cardiovascular risk factors, plasma ferritin concentrations, medication use, and other potential confounders.

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Keywords Hepcidin, Ferritin, Type 2 diabetes, Mortality, Cardiovascular events

Introduction

Over 40 years ago, Dr. Sullivan formulated the "iron hypothesis" for the first time, stating that higher concentrations of stored iron may promote the development of cardiovascular diseases [1]. Subsequently, epidemiological studies assessed the associations of circulating iron, total iron binding capacity, transferrin receptor, and ferritin concentrations with atherosclerosis. In this context, the strongest evidence supporting the "iron hypothesis" was the significant association between higher plasma ferritin concentrations and increased risk of adverse cardiovascular outcomes [2–4].

Hepcidin is a peptide hormone produced by the liver that plays a crucial role in iron metabolism, inhibiting the activity of the cellular iron exporter ferroportin 1 [5, 6]. As a result, intestinal iron absorption is reduced, and iron is stored in macrophages and hepatocytes [5, 6]. Hepcidin is mainly produced by hepatocytes and is increased during iron overload, infections, inflammation, chronic kidney disease, obesity-related metabolic disorders, and chronic liver diseases [5–8]. Some population-based cohort studies reported significant associations between higher circulating hepcidin concentrations and markers of subclinical atherosclerosis, such as increased aortic stiffness and prevalence of carotid atherosclerotic plaques [9–11].

Current evidence about the relationship between circulating hepcidin concentrations and the risk of hard clinical outcomes (such as mortality, acute myocardial infarction, or heart failure) in the general population and patients with established ischemic heart disease is limited and often conflicting [3, 12–16]. Notably and more importantly, no current information is available about the relationship between circulating hepcidin concentrations and the risk of overall mortality and cardiovascular events in people with type 2 diabetes mellitus (T2DM), who are a group of patients at high risk of developing major adverse cardiovascular events, and in whom cardiovascular risk is often difficult to predict. We believe this topic is of clinical relevance, as circulating hepcidin concentrations could be a useful and reliable prognosticator of future mortality and adverse cardiovascular events in this patient population.

Therefore, in this exploratory prospective study, we aimed to examine the association between circulating hepcidin concentrations and the risk of mortality and nonfatal cardiovascular events in a cohort of adult outpatients with established T2DM.

Methods

Participants

We followed for a median period of 55.6 months [interquartile range (IQR): 52.1–81.7 months] 253 adult individuals with established T2DM who consecutively attended our diabetes outpatient service during a 6-month period. We excluded patients with: (a) significant alcohol consumption (defined as >20 g of alcohol per day) and other known causes of chronic liver diseases (e.g., virus, drugs, or autoimmunity); (b) prior history of cirrhosis of any etiology, active cancer, and end-stage renal disease (defined as estimated glomerular filtration rate < 15 mL/min/1.73 m^2 or chronic dialysis); (c) chronic use of potentially hepatotoxic medications, such as non-steroidal anti-inflammatory drugs, steroids, tamoxifen, amiodarone, methotrexate or use of hormone replacement therapy (for women only); and (d) treatment with insulin. No participants had chronic blood losses or chronic intestinal diseases, were chronically treated with blood transfusions or assumed iron supplementation. Approximately half of the study participants (n = 153) have been included in a previously published study of patients undergoing liver ultrasonography and transient elastography to diagnose metabolic dysfunction-associated steatotic liver disease [17].

The local Ethics Committee (Comitato Etico per la Sperimentazione Clinica delle Province di Verona e Rovigo; Prog. #2004CESC and #1399CESC) approved the study protocol. All participants gave their written informed consent for participation in this research.

Clinical and laboratory data

Body mass index (BMI) was measured as kilograms divided by the square of height in meters. Waist circumference was measured at the midpoint between the lowest rib and the iliac crest. Blood pressure was measured with a standard sphygmomanometer after the subject had been seated quietly for at least 5 min. Subjects were considered to have hypertension if their blood pressure was ≥140/90 mmHg or if they were taking any antihypertensive agents.

Venous blood samples were collected in the morning after an overnight fast. Complete blood count, glucose, lipids, liver enzymes, creatinine, high-sensitivity C-reactive protein (hs-CRP) and other biochemical blood parameters were measured using standard laboratory procedures at the Central Laboratory of the Verona Integrated University Hospital, using relative reference techniques and a Cobas® 8000 modular analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Hemoglobin A1c (HbA1c) was measured using the high-performance

liquid chromatography (HPLC) analyzer Tosoh-G7 (Tosoh Bioscience Inc., Tokyo, Japan). Insulin concentration was measured using a chemiluminescent immunoassay (LIAISON, Diasorin, Saluggia, Italy). The homeostasis model assessment (HOMA-IR) score was used to estimate insulin resistance. Glomerular filtration rate (eGFR) was estimated using the CKD-EPI study equation [18]. Urinary albumin excretion was assessed with an immuno-nephelometric assay (Beckman-Coulter IMMAGE; Beckman-Coulter Instruments, Fullerton, CA, USA) on a morning spot urine sample and expressed as the albumin-to-creatinine ratio (ACR); abnormal albuminuria was defined as urinary ACR ≥30 mg/mmol. Chronic kidney disease (CKD) was defined as $eGFR_{CKD-EPI}$ <60 ml/min/1.73 m^2 and/or urinary ACR ≥30 mg/mmol. The FIB-4 index (i.e., a widely used non-invasive biomarker of advanced liver fibrosis) was calculated using the following equation: age ×AST (IU/L)/platelet count ($\times 10^9$/L) ×√ALT (IU/L) [19]. A FIB-4 cut-off >1.3 was suggestive of significant liver fibrosis [17]. A pre-existing history of ischemic heart disease (IHD) or ischemic stroke was assessed as a documented history of myocardial infarction, angina pectoris, coronary revascularization procedures, or ischemic stroke. A pre-existing history of heart failure (HF) or permanent atrial fibrillation (AF) was based on medical history and medical chart reviews. The presence of diabetic retinopathy of any degree (diagnosed with fundoscopy after pupillary dilation) was also recorded.

Measurements of plasma iron, transferrin, ferritin and hepcidin concentrations

Details about the measurement of plasma concentrations of iron, transferrin, ferritin, and hepcidin were published elsewhere [17]. Briefly, blood samples for measurement of plasma iron, transferrin, ferritin and hepcidin were collected into lithium heparin tubes and centrifuged after they arrived in the laboratory. Plasma samples were then stored at -80 °C until analysis. An expert laboratory technician, who was blinded to participants' clinical details, performed the measurements of plasma iron storage biomarkers. These assays, except for hepcidin, were performed by the fully automated analyzer Cobas® 8000 using the following methods: ferritin by electrochemiluminescence (ECLIA), transferrin by immunoturbidimetry and iron by photometry (with chromogenic agent ferrozine). The transferrin saturation was calculated using the following formula: transferrin saturation [%] = plasma iron [μmol/L]/plasma transferrin [g/L] × 3.984. All kits needed for these tests were purchased from Roche Diagnostics (Monza, Italy).

Plasma hepcidin concentration was assessed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Hepcidin-25 standards, both native and isotopic-labeled internal standards (Asp-Thr-His-[13C9,15 N] Phe-Pro-Ile-Cys-Ile-[13C9,15 N] Phe-Cys-Cys-[15 N]Gly-Cys- Cys-His-Arg-Ser-Lys-Cys-Gly-Met-Cys-Cys-Lys-Thr (Mr2810.2)), were purchased from Peptide International (Louisville, Kentucky, USA). Samples were treated by solid-phase extraction using Oasis hydrophilic-lipophilic balanced reversed-phase cartridges (Waters, Milan, Italy). High-performance liquid chromatography (HPLC) was performed using an X-Terra MS C182.5 μm (Waters), and detection was obtained using a Triple Quad LC-MS/MS (Agilent Technologies, Santa Clara, CA, USA).

Primary outcome of the study

The primary study outcome was ascertained by medical record reviews and phone questionnaires in April 2024. Specifically, the primary composite outcome of the study was defined as the occurrence of all-cause mortality and incident cardiovascular outcomes, such as nonfatal myocardial infarction, permanent AF, ischemic stroke or new hospitalization for HF during a median follow-up of 55.6 months (IQR 52.1–81.7 months), corresponding to 13,635 person-months of follow-up.

Statistical analysis

Continuous variables were expressed as means ± SD or medians and inter-quartile ranges (IQRs), whereas categorical variables were expressed as relative percentages. Differences in baseline clinical and biochemical characteristics of participants stratified by baseline hepcidin tertiles were tested by the chi-squared test for categorical variables, the one-way ANOVA for normally distributed continuous variables, and the Kruskal-Wallis test for non-normally distributed variables. Baseline clinical and biochemical differences among participants stratified by primary composite outcome status at follow-up were tested by the chi-squared test for categorical variables, the Student's t-test for normally distributed continuous variables, and the Mann-Whitney test for non-normally distributed variables.

The Kaplan–Meier survival curves were used to test the risk of developing the primary composite outcome over the follow-up period in participants stratified either by baseline hepcidin tertiles (3rd tertile vs. 1st and 2nd tertiles combined) or by baseline ferritin tertiles (3rd tertile vs. 1st and 2nd tertiles combined). The Cox proportional-hazards models assessed the independent association between baseline plasma hepcidin tertiles (3rd tertile vs. 1st and 2nd tertiles combined) and the risk of developing the primary composite outcome at follow-up. Specifically, we performed three Cox proportional hazards models. The first model was unadjusted, and the second model was adjusted for age, sex, prior IHD, prior HF, eGFR and logarithmically transformed plasma ferritin

concentrations. In this regression model, we included as covariates the variables that significantly differed at baseline between those who developed the primary study outcome at follow-up and those who did not. The third regression model was adjusted for age, sex, BMI, smoking status, hypertension (defined as blood pressure ≥ 140/90 mmHg and/or drug treatment), statin use, antiplatelet medication use, and logarithmically transformed plasma hs-CRP and ferritin concentrations. The inclusion of covariates in this latter Cox regression model was based on biological plausibility.

A *P*-value < 0.05 was considered statistically significant. Statistical analyses were performed using R 4.4.0 (R Core Team 2024, R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/) and Python 3.12.3.

Results

Among the 213 adult outpatients with established T2DM included in the study (45.5% women, mean age 69 ± 10 years; BMI 28.7 ± 4.7 kg/m^2; HbA1c 52 ± 7 mmol/mol; median diabetes duration 11 [IQR 6–19] years), 42 patients developed the primary composite outcome during a median follow-up of 55.6 months. Of these 42 events, 12 were all-cause deaths, 9 were incident nonfatal myocardial infarctions, 12 were incident permanent AF, 4 were incident nonfatal strokes, and 5 were new hospitalizations for HF.

Figure 1 shows the distribution of baseline plasma hepcidin concentrations (including the tertile cut points) in the whole sample of participants.

Table 1 summarizes the baseline clinical and biochemical characteristics of participants stratified by baseline hepcidin tertiles. Compared to those in the 1st tertile, who had median plasma hepcidin concentrations of 1.04 (IQR 0.50–1.95) nmol/L, patients in the 3rd tertile, who had a median plasma hepcidin concentrations of 7.72 (IQR 6.37–10.4) nmol/L, had a shorter diabetes duration, lower plasma transferrin concentrations, and higher circulating concentrations of hs-CRP, iron, ferritin, transferrin saturation, and hemoglobin. Sulfonylureas and anti-platelet agents were used less frequently in those belonging to the 3rd hepcidin tertile. Notably, at baseline, the proportion of patients with the primary composite outcome at follow-up was significantly greater in those belonging to the 3rd hepcidin tertile (50%) than those in the 1st or 2nd hepcidin tertiles (21.4% and 28.6%). Conversely, age, sex, adiposity measures, blood pressure, smoking, glucose, HbA1c, HOMA-IR score, lipids, eGFR, albuminuria, liver enzymes, proportion of those with

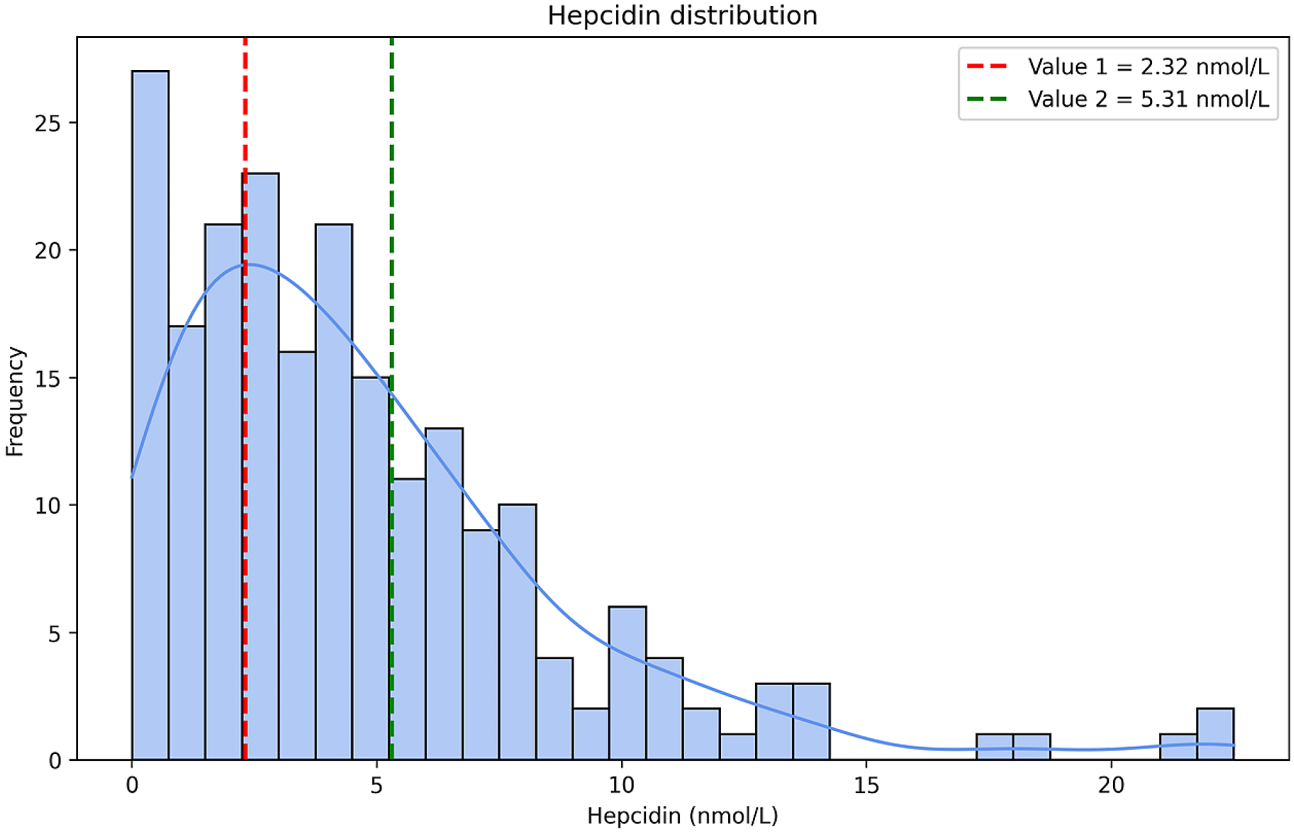

Fig. 1 Distribution of baseline plasma hepcidin concentrations, including the tertile cut points (marked with red and green dotted lines), among the study participants

Table 1 Baseline clinical and biochemical characteristics of participants stratified by plasma hepcidin tertiles

	1st hepcidin tertile (*n* = 71) Median: 1.04 (IQR 0.50–1.95) nmol/L	**2nd hepcidin tertile (*n* = 70) Median: 3.81 (IQR 3.01-4-42) nmol/L**	**3rd hepcidin tertile (*n* = 72) Median: 7.72 (IQR 6.37–10.4) nmol/L**	***P*-value**
Age (years)	70 ± 9	70 ± 9	68 ± 11	0.105
Male sex (%)	52.1	47.1	63.9	0.119
BMI (kg/m^2)	28.3 ± 5.2	28.5 ± 4.8	29.1 ± 4.0	0.538
Waist circumference (cm)	99.4 ± 12.3	102.4 ± 14.5	101.7 ± 10.3	0.349
Diabetes duration (years)	16.0 (10.0-22.8)	10.5 (6.0–17.0)	8.0 (3.75–14.3)	**< 0.001**
Current smokers (%)	18.3	15.7	11.1	0.319
Systolic blood pressure (mmHg)	136 ± 18	134 ± 19	133 ± 18	0.671
Diastolic blood pressure (mmHg)	76 ± 9	75 ± 10	76 ± 9	0.927
Hemoglobin (g/L)	135 ± 13	136 ± 13	142 ± 14	**0.007**
Iron (µmol/L)	12.7 (9.9–16.9)	14.6 (11.7–17.8)	15.2 (11.9–18.4)	**0.035**
Ferritin (µg/L)	36.8 (21.0-71.1)	87.8 (55.5-136.9)	158.2 (114.3-251.6)	**< 0.001**
Transferrin (g/L)	2.9 (2.6–3.2)	2.6 (2.4–2.9)	2.6 (2.3–2.8)	**< 0.001**
Transferrin saturation (%)	17.9 (12.8–22.7)	23.0 (17.6–28.1)	22.8 (17.6–28.6)	**< 0.001**
Fasting glucose (mg/dL)	128 ± 26	130 ± 26	129 ± 33	0.943
Hemoglobin A1c (mmol/mol)	53 ± 9	54 ± 10	51 ± 7	0.169
Total cholesterol (mg/dL)	151 ± 37	154 ± 29	157 ± 38	0.591
LDL-cholesterol (mg/dL)	74 ± 32	77 ± 26	80 ± 34	0.492
HDL-cholesterol (mg/dL)	53 ± 15	52 ± 13	52 ± 15	0.865
Triglycerides (mg/dL)	109 (82–150)	110 (86–161)	111 (84–149)	0.826
Fasting insulin (mUI/L)	5.5 (3.3–9.1)	7.6 (3.8–11.1)	5.6 (3.6–9.8)	0.349
HOMA-IR score	1.69 (1.02–2.92)	2.17 (1.23–3.50)	1.87 (1.03–3.21)	0.383
AST (IU/L)	22 (18–28)	23 (20–27)	24 (20–27)	0.816
ALT (IU/L)	12 (9–17)	13 (10–16)	13 (10–16)	0.736
GGT (IU/L)	17 (13–27)	23 (14–37)	20 (14–30)	0.116
FIB-4 index > 1.3 (%)	88.7	84.3	81.9	0.515
hs-C-reactive protein (mg/L)	1.0 (0.4–2.1)	1.1 (0.7–2.4)	1.5 (0.7–3.9)	**0.043**
Creatinine (µmol/L)	77.4 ± 21.6	82.6 ± 42.9	77.6 ± 21.2	0.530
$eGFR_{CKD-EPI}$ (mL/min/1.73 m^2)	78.6 ± 17.6	81.1 ± 17.8	81.9 ± 18.1	0.199
Abnormal albuminuria (%)	20.0	17.1	25.8	0.364
Hypertension (%)	83.1	82.9	81.9	0.982
Prior IHD (%)	16.9	14.3	19.4	0.714
Prior heart failure (%)	1.4	1.4	1.4	0.998
Prior atrial fibrillation	7.0	2.9	1.4	0.183
Prior ischemic stroke (%)	0	5.7	2.8	0.122
Diabetic retinopathy (%)	15.5	15.7	11.3	0.590
Chronic kidney disease (%)	12.7	20.0	18.0	0.485
Metformin users (%)	83.1	77.1	81.9	0.636
Sulfonylurea users (%)	29.6	37.1	18.1	**0.039**
Pioglitazone users (%)	12.7	4.3	8.3	0.201
DPP-4 inhibitor users (%)	30.9	24.3	18.1	0.198
GLP-1 receptor agonist users (%)	23.9	14.3	19.4	0.347
SGLT-2 inhibitor users (%)	8.5	11.4	11.1	0.815
Anti-platelet users (%)	67.6	48.6	37.5	**0.001**
Anti-coagulant users (%)	5.6	2.9	5.6	0.675
Beta-blocker users (%)	28.2	41.4	36.1	0.252
ARB/ACE-inhibitor users (%)	64.8	65.7	63.9	0.974
CCB users (%)	19.7	30.0	20.8	0.287
Diuretic users (%)	38.1	37.1	29.2	0.472

Table 1 (continued)

	1st hepcidin tertile ($n=71$) Median: 1.04 (IQR 0.50–1.95) nmol/L	2nd hepcidin tertile ($n=70$) Median: 3.81 (IQR 3.01-4-42) nmol/L	3rd hepcidin tertile ($n=72$) Median: 7.72 (IQR 6.37–10.4) nmol/L	*P*-value
Statin users (%)	78.9	78.6	79.2	0.996
Primary composite outcome[§] (%)	28.6	21.4	50.0	**0.038**

Sample size: $n=213$. Data are expressed as means ± SD, medians and interquartile ranges (IQRs) (in parenthesis) or percentages. Differences among the three patient groups were tested by the chi-squared test for categorical variables, the one-way ANOVA for normally distributed continuous variables, and the Kruskal-Wallis test for non-normally distributed variables (i.e., diabetes duration, ferritin, iron, transferrin, hepcidin, triglycerides, insulin, HOMA-IR score, C-reactive protein and liver enzymes). For the sake of clarity, significant p-values are highlighted in bold

[§]Primary composite outcome included all-cause mortality or incident cardiovascular events (inclusive of nonfatal myocardial infarction, permanent atrial fibrillation, ischemic stroke or new hospitalization for heart failure)

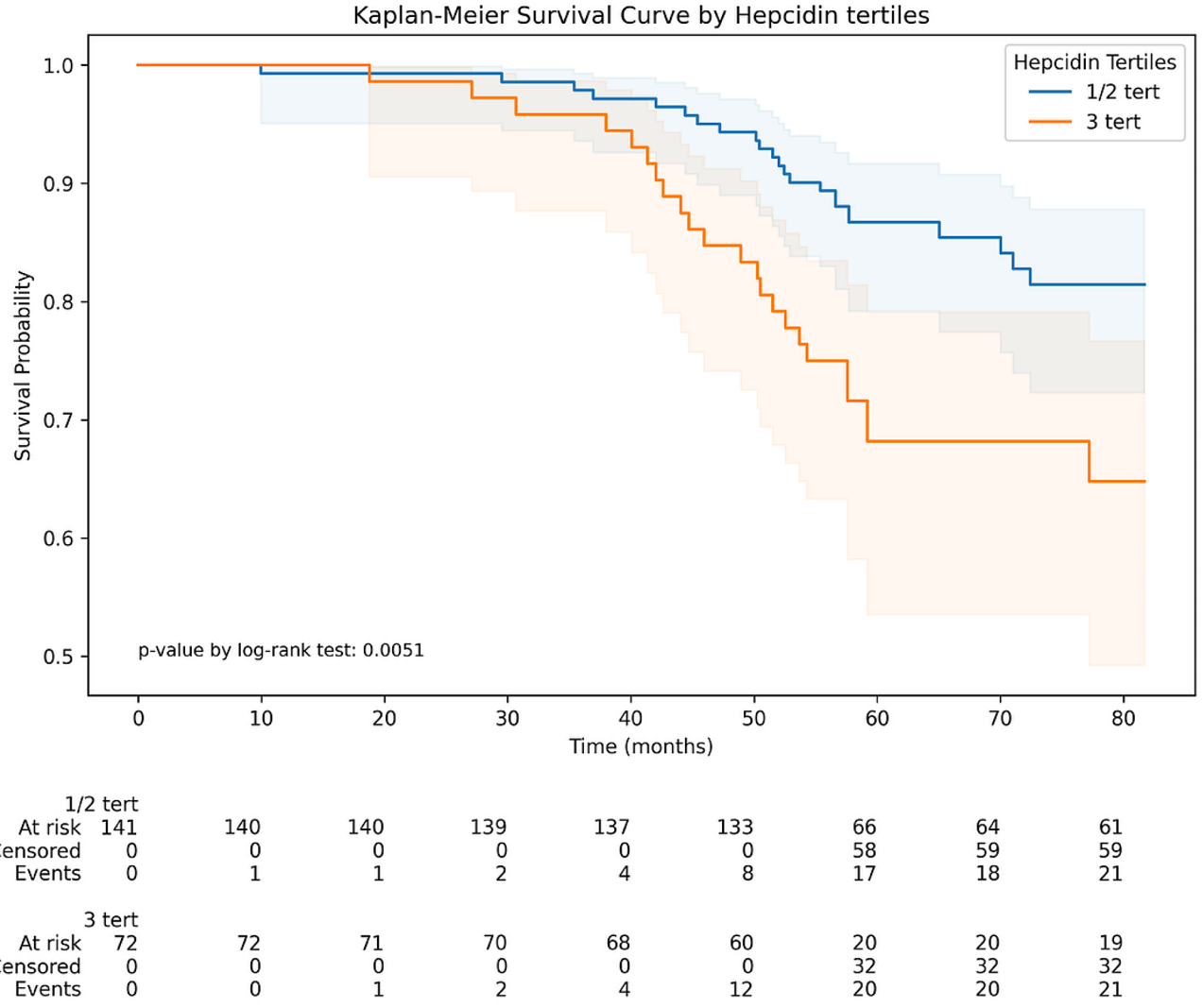

Fig. 2 Kaplan-Meier survival estimates (with 95% confidence intervals, as shaded areas) of the risk of developing the primary composite outcome over the follow-up period in patients with T2DM stratified by baseline plasma hepcidin tertiles (3rd tertile vs. 1st and 2nd tertiles combined)

FIB-4 index > 1.3, hypertension, diabetic retinopathy and previous histories of IHD, HF, permanent AF or ischemic stroke, as well as use of glucose-lowering agents (except for sulfonylureas), anti-hypertensive drugs and statins did not significantly differ among the three patient groups.

The Kaplan-Meier survival estimates of the risk of developing the primary composite outcome over the follow-up in participants stratified by baseline hepcidin tertiles are shown in Fig. 2. Patients in the 3rd hepcidin tertile had a substantially higher risk of developing the

primary composite outcome during the follow-up period than those in the 1st and 2nd hepcidin tertiles combined (P-value by log-rank test: 0.005).

Table 2 shows the baseline clinical and biochemical characteristics of participants stratified by the primary composite outcome status at follow-up. At baseline, patients who developed the primary composite outcome at follow-up were more likely to be older and had significantly higher plasma concentrations of hepcidin, ferritin, and creatinine and lower eGFR values than those who did not. Previous histories of IHD and HF were also higher in patients experiencing the primary composite outcome at follow-up than those who did not. The other baseline clinical and biochemical characteristics (including adiposity measures, blood pressure, diabetes duration, glycemic control, plasma lipids and hemoglobin) did not differ significantly between the two patient groups.

The associations between baseline plasma hepcidin tertiles (3rd tertile vs. 1st and 2nd tertiles combined) and the risk of developing the primary composite outcome of the study, using Cox proportional hazards models, are reported in Table 3. In the unadjusted regression model, patients belonging to the 3rd hepcidin tertile had a 2.3-fold increased risk of developing the primary composite outcome compared to those belonging to the 1st and 2nd tertiles combined (unadjusted HR 2.32, 95%CI 1.27–4.26; $p=0.007$). Adjustment for age, sex, prior IHD, prior HF, eGFR and logarithmically transformed plasma ferritin concentrations did not weaken the significant association between baseline hepcidin tertiles and the risk of developing the primary composite outcome (model 1: adjusted HR 2.26, 95% CI 1.15–4.46; $p=0.018$). In this adjusted model, other independent predictors of the primary composite outcome were older age, male sex and previous history of HF. As reported in adjusted model 2, the risk of developing the primary composite outcome in patients belonging to the 3rd hepcidin was ~2.5-fold greater than that observed in those belonging to the 1st and 2nd tertiles combined, even after adjustment for age, sex, BMI, smoking history, hypertension, statin use antiplatelet drug use, and logarithmically transformed plasma hs-CRP and ferritin concentrations (adjusted HR 2.53, 95%CI 1.27–5.03; $p=0.008$). In this latter adjusted model (where covariates were chosen based on their biological plausibility), older age and male sex were also independently associated with a higher risk of developing the primary composite outcome. Plasma ferritin concentrations were not independently associated with the primary composite outcome in both adjusted regression models. Almost identical results were observed even when patients with a previous history of IHD were excluded from the statistical analysis (data not shown).

Supplementary Fig. 1 shows the Kaplan-Meier survival estimates of the risk of developing the primary composite outcome over the follow-up in participants stratified by baseline plasma ferritin tertiles. Patients in the 3rd ferritin tertile had a significantly higher risk of developing the primary composite outcome than those in the 1st and 2nd ferritin tertiles combined (p-value by log-rank test: 0.011).

Discussion

To our knowledge, this is the first prospective cohort study examining the association between baseline plasma hepcidin concentrations and the long-term risk of all-cause mortality and nonfatal cardiovascular events in ambulatory patients with established T2DM. The main and novel findings of our exploratory cohort study are as follows: (a) higher baseline plasma hepcidin concentrations were significantly associated with a 2.3-fold increased risk of developing the primary composite outcome of the study in patients with T2DM followed for a median period of 55.6 months (IQR 52.1–81.7 months); (b) this association remained statistically significant even after adjustment for common cardiovascular risk factors, medication use, and other potential confounders (also including plasma hs-CRP and ferritin concentrations); and (c) in unadjusted Cox regression analysis, higher baseline plasma ferritin concentrations were associated with a higher risk of developing the primary composite outcome, confirming the results of previously published cohort studies [2–4].

Epidemiological studies have examined the associations of circulating iron, total iron binding capacity, transferrin receptor, and ferritin concentrations with the risk of mortality and nonfatal cardiovascular events in the general adult population and patients with established IHD. While the strongest evidence concerned the association between higher plasma ferritin concentrations and the risk of fatal and nonfatal cardiovascular events [2–4], the currently available information regarding a possible association between circulating hepcidin concentrations and the risk of adverse cardiovascular outcomes is limited and conflicting. In a prospective study of 759 patients with acute coronary syndrome (ACS) and 526 patients with stable IHD followed for a mean period of 4.1 years, Li et al. reported that higher baseline plasma hepcidin concentrations were independently associated with higher all-cause and cardiovascular mortality in ACS patients but not in those with stable IHD [13]. In another prospective study always focusing on secondary cardiovascular prevention in 3,423 patients with stable IHD, Zeller et al. did not detect any significant association between baseline hepcidin concentrations and risk of adverse cardiovascular outcomes [15]. In contrast, in a prospective study of 811 patients with stable IHD, Ruhe et al. reported that baseline hepcidin concentrations were independently associated with a lower risk of cardiovascular mortality

Table 2 Baseline clinical and biochemical characteristics of participants stratified by primary composite outcome status at follow-up

	Absence of primary composite outcome at follow-up (*n* = 171)	Presence of primary composite outcome at follow-up (*n* = 42)	*P*-value
Age (years)	68 ± 9	73 ± 10	**0.005**
Male sex (%)	52.6	61.9	0.279
BMI (kg/m^2)	28.5 ± 4.8	29.2 ± 4.6	0.414
Waist circumference (cm)	100.3 ± 12.8	104.4 ± 10.8	0.062
Diabetes duration (years)	11 (6–19)	12 (7–18)	0.987
Current smokers (%)	15.8	11.9	0.202
Systolic blood pressure (mmHg)	135 ± 18	132 ± 18	0.300
Diastolic blood pressure (mmHg)	76 ± 9	74 ± 10	0.220
Hemoglobin (g/L)	137 ± 13	140 ± 14	0.261
Iron (µmol/L)	14.0 (10.9–17.8)	15.1 (11.9–17.4)	0.237
Ferritin (µg/L)	81.2 (41.1-142.9)	123.7 (71.8-201.5)	**0.027**
Transferrin (g/L)	2.7 (2.5-3.0)	2.7 (2.3–3.2)	0.977
Transferrin saturation (%)	20.7 (16.1–25.9)	21.9 (17.8–28.9)	0.269
Hepcidin (nmol/L)	3.5 (1.9–6.12)	5.0 (2.0-7.2)	**0.047**
Hepcidin tertiles			**0.039**
1st tertile	83.1	16.9	
2nd tertile	87.1	12.8	
3rd tertile	70.8	29.2	
Fasting glucose (mg/dL)	130 ± 30	126 ± 21	0.359
Hemoglobin A1c (mmol/mol)	53 ± 9	51 ± 8	0.121
Total cholesterol (mg/dL)	155 ± 35	147 ± 35	0.185
LDL-cholesterol (mg/dL)	79 ± 31	71 ± 31	0.174
HDL-cholesterol (mg/dL)	53 ± 15	51 ± 14	0.384
Triglycerides (mg/dL)	109 (82–152)	114 (92–159)	0.322
Fasting insulin (mUI/L)	6.2 (3.4–10.0)	7.4 (3.6–9.9)	0.423
HOMA-IR score	1.9 (1.1–3.21)	2.0 (1.2–3.30)	0.561
AST (IU/L)	23 (20–27)	23 (19–31)	0.354
ALT (IU/L)	13 (10–17)	12 (9–16)	0.937
GGT (IU/L)	19 (14–30)	21 (13–28)	0.729
FIB-4 index > 1.3 (%)	83.0	93.0	0.116
hs-C-reactive protein (mg/L)	1.2 (0.6–2.6)	1.4 (0.5–3.5)	0.957
Creatinine (µmol/L)	76.4 ± 22	90.4 ± 51	**0.007**
$eGFR_{CKD-EPI}$ (mL/min/1.73 m^2)	80.7 ± 17	74.5 ± 19	**0.005**
Abnormal albuminuria (%)	18.5	31.6	0.079
Hypertension (%)	80.7	90.5	0.134
Prior IHD (%)	13.5	30.9	**0.007**
Prior heart failure (%)	0.6	4.8	**0.039**
Prior atrial fibrillation	3.5	4.8	0.702
Prior ischemic stroke (%)	2.9	2.4	0.848
Diabetic retinopathy, any degree (%)	14.0	14.6	0.883
Chronic kidney disease (%)	15.8	21.4	0.382
Metformin users (%)	82.4	73.8	0.203
Sulfonylurea users (%)	29.2	23.8	0.483
Pioglitazone users (%)	9.9	2.4	0.115
DPP-4 inhibitor users (%)	22.8	30.9	0.271
GLP-1 receptor agonist users (%)	21.1	11.9	0.178
SGLT-2 inhibitor users (%)	10.5	9.5	0.848
Anti-platelet users (%)	49.1	59.5	0.227
Beta-blocker users (%)	31.6	50.0	**0.046**
ARB/ACE-inhibitor users (%)	62.6	73.8	0.172
CCB users (%)	23.9	21.4	0.727

Table 2 (continued)

	Absence of primary composite outcome at follow-up (*n* = 171)	Presence of primary composite outcome at follow-up (*n* = 42)	*P*-value
Diuretic users (%)	35.1	33.3	0.831
Statin users (%)	78.4	80.9	0.713

Sample size: *n* = 213. Data are expressed as means ± SD, medians and interquartile ranges (IQRs) (in parenthesis) or percentages. Differences among the two patient groups were tested by the chi-squared test for categorical variables, the Student's t-test for normally distributed continuous variables, and the Mann-Whitney test for non-normally distributed variables (i.e., diabetes duration, ferritin, iron, transferrin, hepcidin, triglycerides, fasting insulin, HOMA-IR score, C-reactive protein and liver enzymes). For the sake of clarity, significant p-values are highlighted in bold

Table 3 Associations between plasma hepcidin tertiles (3rd tertile vs. 1st and 2nd tertiles combined) and the risk of developing the primary composite outcome

	Hazard Ratio(s)	95% CI(s)	*P*-value
Unadjusted Cox regression model			
Plasma hepcidin tertiles			
1st and 2nd tertiles combined (*n* = 141)	*Ref.*	*Ref.*	-
3rd tertile (*n* = 72)	2.32	1.27–4.26	**0.007**
Adjusted Cox regression model 1[§]			
Plasma hepcidin tertiles			
1st and 2nd tertiles combined (*n* = 141)	*Ref.*	*Ref.*	-
3rd tertile (*n* = 72)	2.26	1.15–4.46	**0.018**
Age (years)	1.05	1.01–1.10	**0.013**
Sex (men vs. women)	3.45	1.45–8.21	**0.005**
Prior IHD (yes vs. no)	1.83	0.91–3.71	0.090
Prior HF (yes vs. no)	6.12	1.28–29.3	**0.023**
$eGFR_{CKD-EPI}$ (mL/min/1.73 m^2)	0.99	0.97–1.01	0.240
(Ln) Plasma ferritin (ug/L)	1.01	0.98–1.02	0.201
Adjusted Cox regression model 2[#]			
Plasma hepcidin tertiles			
1st and 2nd tertiles combined (*n* = 141)	*Ref.*	*Ref.*	-
3rd tertile (*n* = 72)	2.53	1.27–5.03	**0.008**
Age (years)	1.07	1.03–1.11	**0.001**
Sex (men vs. women)	4.37	1.81–10.6	**0.001**
BMI (kg/m^2)	1.06	0.99–1.33	0.121
Smoking status (yes vs. no)	1.43	0.53–3.84	0.475
Arterial hypertension (yes vs. no)	1.25	0.42–3.72	0.685
Statin use (yes vs. no)	1.04	0.47–2.31	0.928
Antiplatelet medication use (yes vs. no)	1.36	0.70–2.63	0.362
(Ln) hs-C-reactive protein (mg/L)	0.95	0.87–1.02	0.154
(Ln) Plasma ferritin (ug/L)	1.00	0.99–1.01	0.206

Sample size, *n* = 213. Data are expressed as hazard ratio(s) and 95% confidence intervals assessed by Cox proportional hazards models. The dependent variable of all these Cox regression models was a primary composite outcome, inclusive of all-cause mortality or incident cardiovascular events (including incident nonfatal myocardial infarction, permanent atrial fibrillation, ischemic stroke or new hospitalization for heart failure)

[§]Covariates included in adjusted Cox regression model 1 were chosen as potential confounding variables based on statistical associations in univariable analyses (as reported in Table 2)

[#]Covariates included in adjusted Cox regression model 2 were chosen as potential confounding variables based on biological plausibility

during a median follow-up of 4 years [16]. Similarly, in another prospective study enrolling 1,480 patients with stable CAD, Grammer et al. reported that baseline hepcidin concentrations were associated with a lower risk of all-cause and cardiovascular mortality during a median of 9.9 years [3]. Finally, in a population-based cohort study involving 6,386 adult individuals followed for a median of 8.3 years, Klip et al. reported that baseline hepcidin concentrations were significantly associated with a higher risk of all-cause mortality and cardiovascular events in women but not in men, after adjustment for common cardiovascular risk factors and other potential confounders [2].

Collectively, therefore, the results of our prospective study confirm and further expand previous findings in outpatients with T2DM (~ 85% of whom were in primary prevention of cardiovascular disease), showing that higher baseline hepcidin concentrations were associated with a more than double risk of all-cause mortality or incident nonfatal cardiovascular events after adjustment for traditional cardiovascular risk factors, medication use, plasma hs-CRP and ferritin concentrations, and other potential confounders.

Putative mechanisms underpinning the association between elevated hepcidin concentrations and risk of adverse cardiovascular outcomes

The precise biological mechanisms underpinning the association between elevated circulating hepcidin concentrations and the risk of mortality and nonfatal cardiovascular events in people with T2DM are not fully understood. Given the well-known association between plasma ferritin concentrations, hemochromatosis and dilated cardiomyopathy [20], it is interesting to note that there were no significant associations between plasma hepcidin concentrations and indices of liver function or advanced fibrosis. That said, it is important to remember that the liver is the primary source of hepcidin production in humans [21]. Metabolic dysfunction-associated steatotic liver disease (MASLD) is the most common cause of chronic liver disease in patients with T2DM, and is significantly associated with adverse cardiovascular outcomes [22, 23] and higher circulating levels of iron storage biomarkers (especially in the presence of metabolic dysfunction-associated steatohepatitis [MASH]

with increasing levels of liver fibrosis) [17, 24–26]. It is, therefore, reasonable to hypothesize that the presence of MASH with varying amounts of liver fibrosis could be a mediating factor in the association we observed between elevated circulating hepcidin concentrations and the risk of mortality and nonfatal cardiovascular events in people with T2DM. Although we had data on FIB-4 index (i.e., a non-invasive biomarker of advanced liver fibrosis), we did not perform a liver biopsy or magnetic resonance elastography for accurately staging liver fibrosis. Liver biopsy assessment is difficult to justify in individuals with fairly normal serum liver enzyme levels (such as those observed in most of our participants). Speculatively, the most obvious explanation for our findings is that this association is an epiphenomenon of shared cardiovascular risk factors and important comorbidities. However, it should be noted that in our study, the significant association between baseline hepcidin concentrations and the long-term risk of all-cause mortality and nonfatal cardiovascular events persisted after adjusting for established cardiovascular risk factors, medication use (also including statin and antiplatelet medication use), plasma hs-CRP, plasma ferritin and other potential confounding factors. Therefore, although additional mechanistic studies are required, it is possible to hypothesize that higher circulating hepcidin concentrations may play a role in the development of cardiovascular mortality and events. Experimentally, hepcidin increases iron deposition in macrophages within atherosclerotic plaques, exacerbating lipid peroxidation, foam cell formation, and progression of atherosclerosis [13, 27]. Foam cells may promote the pro-inflammatory microenvironment of atherosclerotic plaques by secreting multiple proinflammatory cytokines, reactive oxygen species, and proteases [28]. For example, Valenti et al. reported that serum hepcidin and macrophage iron are significantly associated with monocyte chemo attractant protein-1 (MCP-1) release and vascular damage in patients with metabolic syndrome alterations [29]. Studies also showed that hepcidin deficiency is associated with reduced intracellular macrophage iron and a non-foam cell phenotype [30, 31]. Hepcidin may also promote the production of reactive oxygen species, thus increasing lipid peroxidation (thereby resulting in ferroptosis) and decreasing cholesterol efflux, a mechanism that may contribute to plaque destabilization [13, 32]. Ferroptosis is an iron-dependent form of regulated cell death that has become increasingly recognized as an important process mediating the pathogenesis and progression of numerous cardiovascular diseases, including coronary atherosclerosis, heart failure, arrhythmia and diabetic cardiomyopathy [33]. Increased circulating ferritin concentrations have also been associated with iron overload in adipose tissue, which may worsen insulin resistance and promote the release of various pro-inflammatory cytokines, thus further favoring plaque formation and destabilization [34, 35]. Collectively, therefore, the evidence from experimental data suggests that hepcidin and ferritin may play a detrimental role in the development and progression of atherosclerosis, possibly via several mechanisms, including enhanced oxidative stress, production of pro-inflammatory cytokines and increased foam cell formation [6]. However, further mechanistic studies are needed. The interpretation of experiments based on acute actions should be taken with some caution, particularly when extrapolated to mild chronic inflammatory states, which could prevail in a substantial number of participants who took part in the present study (as reflected by their plasma hs-CRP concentrations). Given that plasma hepcidin concentrations have yet to be firmly established for mild chronic inflammatory states, as well as the downstream effects of hepcidin on iron status in different organs, it remains to be better elucidated if, at the basal hepcidin concentrations detected in our T2DM patients, the hormone has any physiological effect.

Study limitations and strengths

Our study has some limitations that should be mentioned. First, the observational design of the study did not allow us to establish a cause-effect relationship between plasma hepcidin concentrations and the risk of mortality and nonfatal cardiovascular events. Second, our study included a relatively small cohort of Caucasian individuals with metabolically well-controlled T2DM who regularly attended a diabetes outpatient service. Hence, these results could not be generalizable to other patient groups with T2DM. In addition, a control group of nondiabetic individuals was lacking. Third, we did not perform the HFE (human homeostatic iron regulator protein) genetic analysis and the quantitative measurement of hepatic iron content that would have permitted an accurate quantification of hepatic iron deposition in our patients. Finally, we cannot exclude residual confounding due to unmeasured or unknown risk factors. For example, we did not measure plasma 25-hydroxyvitamin D_3 concentrations among the study participants, but it is known that vitamin D_3 is a potent regulator of the hepcidin-ferroportin axis in humans [36, 37].

Despite these limitations, our study has some important strengths, such as the completeness of the data collection, the consecutive enrolment of the study population, the relatively long duration of follow-up, the adjustment for important confounding factors, and the exclusion of patients with important comorbidities (such as cirrhosis, advanced renal disease or active cancer), as we believe that the inclusion of patients with such comorbidities might have confounded the interpretation of data. Furthermore, we measured plasma hepcidin

concentrations using liquid chromatography-tandem mass spectrometry (LC-MS/MS), which is considered the "gold standard" method for measuring hepcidin-25 concentrations in blood. However, this method is expensive and takes time to obtain results. Although several less expensive and faster laboratory assays have been developed for hepcidin measurements, efforts toward harmonization are ongoing [8].

Conclusions

The results of this exploratory prospective cohort study showed for the first time that higher circulating hepcidin concentrations were significantly associated with a more than double risk of all-cause mortality and nonfatal cardiovascular events in adult outpatients with established T2DM over a median of 55.6 months. This association remained significant after adjustment for traditional cardiometabolic risk factors, plasma ferritin concentrations, medication use, and other potential confounding factors (including plasma hs-CRP concentrations). Hepcidin might, therefore, have a prognostic value in predicting the future risk of adverse cardiovascular outcomes in people with T2DM. However, further prospective and mechanistic studies are required to corroborate our findings in other independent cohorts of patients with T2DM and to better understand the complex mechanisms underpinning the association between iron storage biomarkers and increased risk of cardiovascular mortality and events.

Abbreviations

ACE	Angiotensin-converting-enzyme inhibitor
ARB	Angiotensin II receptor blocker
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMI	Body mass index
CCB	Channel-calcium blocker
DPP-4	Dipeptidyl peptidase-4
e-$GFR_{CKD-EPI}$	Estimated glomerular filtration rate calculated by the CKD-Epidemiology Collaboration equation
FIB-4	Fibrosis 4
GGT	Gamma-glutamyl-transferase
GLP-1	Glucagon-like peptide-1
HOMA-IR	Homeostasis model assessment—insulin resistance
SGLT-2	Sodium/glucose cotransporter-2

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12933-024-02377-x.

Supplementary Material 1—Kaplan-Meier survival estimates (with 95% confidence intervals, as shaded areas) of the risk of developing the primary composite outcome over the follow-up period in patients with T2DM stratified by baseline plasma ferritin tertiles (3rd tertile vs. 1st and 2nd tertiles combined)

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None.

Author contributions

A.M. and G.T. were involved in the conception, design, and conduct of the study. G.T. and D.G. coordinated the data. All authors contributed to data collection. A.M. and G.T. wrote the first draft of the manuscript. A.M. and G.T. conducted the statistical analysis. J.B., C.D.B., L.V. and D.G. contributed to the interpretation of the results. All authors edited, reviewed, and approved the final version of the manuscript. G.T. is the guarantor of this work and, as such, has full access to all the data and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Data availability

The dataset supporting the conclusions of this article is included within the article (and its additional files).

Declarations

Ethics approval and consent to participate

The study protocol was approved by the "Comitato Etico per la Sperimentazione Clinica delle Province di Verona e Rovigo"; Prog. #2004CESC and #1399CESC. All participants gave their written informed consent for participation in this research.

Consent for publication

We agree to the publication of the data; we also declare that the data are original and are not under review elsewhere.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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