Heart Valve Calcification in Patients with Type 2 Diabetes and Nonalcoholic Fatty Liver Disease

Running Title: Valvular calcification and NAFLD in diabetes

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ABSTRACT

Purpose: Aortic valve sclerosis (AVS) and mitral annulus calcification (MAC) are two powerful predictors of adverse cardiovascular outcomes in patients with type 2 diabetes, but the aetiology of valvular calcification is uncertain. Nonalcoholic fatty liver disease (NAFLD) is an emerging cardiovascular risk factor and is very common in type 2 diabetes, but whether NAFLD is associated with valvular calcification in this group of patients is presently unknown.

Methods: We undertook a cross-sectional study of 247 consecutive type 2 diabetic outpatients with no previous history of heart failure, valvular heart diseases (aortic stenosis, mitral stenosis, moderate or severe aortic and mitral regurgitation) or hepatic diseases. Presence of MAC and AVS was detected by echocardiography. NAFLD was diagnosed by ultrasonography.

Results: Overall, 139 (56.3%) patients had no heart valve calcification (HVC-0), 65 (26.3%) patients had one valve affected (HVC-1) and 43 (17.4%) patients had both valves affected (HVC-2). 175 (70.8%) patients had NAFLD and the prevalence of this disease markedly increased in patients with HVC-2 compared with either HVC-1 or HVC-0 (86.1% *vs.* 83.1% *vs.* 60.4%, respectively; $p<0.001$). NAFLD was significantly associated with AVS and/or MAC (unadjusted-odds ratio 3.51, 95%CI 1.89–6.51, $p<0.001$). Adjustments for age, sex, waist circumference, smoking, blood pressure, hemoglobin A1c, LDL-cholesterol, kidney function parameters, medication use and echocardiographic variables did not appreciably weaken this association (adjusted-odds ratio 2.70, 95%CI 1.23-7.38, $p<0.01$).

Conclusions: Our results show that NAFLD is an independent predictor of cardiac calcification in both the aortic and mitral valves in patients with type 2 diabetes.

Key-words: aortic valve sclerosis; heart valve calcification; mitral annulus calcification; nonalcoholic fatty liver disease; type 2 diabetes

List of abbreviations:

AVS, aortic valve sclerosis
BMI, body mass index
eGFR, estimated glomerular filtration rate
HbA1c, hemoglobin A1c
HVC, heart valve calcification
IHD, ischemic heart disease
IVRT, isovolumetric relaxation time
LV, left ventricular
MAC, mitral annulus calcification
NAFLD, nonalcoholic fatty liver disease
SAC, systemic arterial compliance
SVR, systemic vascular resistance

INTRODUCTION

Aortic valve sclerosis (AVS) is present in approximately 30% of adults >65 years of age [1,2]. For decades, this disease was thought to be a passive process of little or no clinical significance in which the valve degenerated with age in association with calcium accumulation. However, AVS shares histopathological and epidemiological features with coronary atherosclerosis [2], and large prospective studies have now documented that AVS is independently associated with increased cardiovascular mortality and morbidity both in the general population and in non-diabetic high-risk patient individuals [3-6]. Mitral annulus calcification (MAC) is common in the elderly (~15%) and is also now known to be associated with adverse cardiovascular outcomes [6-8]. Recently, we have also shown that the presence of AVS and MAC, singly or in combination, is very common in patients with type 2 diabetes (occurring in up to ~45% of patients), and AVS and MAC are associated with an increased risk of all-cause and cardiovascular mortality, independently of established risk factors [9]. Notably, we showed that the combined presence of AVS and MAC is more strongly associated with increased risk of all-cause and cardiovascular mortality than the presence of either AVS or MAC alone [9].

In parallel, nonalcoholic fatty liver disease (NAFLD) has emerged as a public health problem of epidemic proportions worldwide [10]. NAFLD is highly prevalent in people with type 2 diabetes (occurring in up to 70% of patients) [11-13], and is not only associated with liver-related mortality and morbidity, but is also associated with an increased risk of developing ischemic heart disease (IHD), abnormalities of myocardial function and structure, and cardiac arrhythmias (*e.g.*, atrial fibrillation) [14-16]. Preliminary evidence also suggests that NAFLD is associated with the presence of AVS, independently of established cardiovascular risk factors, in both non-diabetic and type 2 diabetic individuals [17,18]. However, to our knowledge, no studies have tested associations between NAFLD and MAC, or associations between NAFLD and AVS, to determine whether similar (or different) associations occur between NAFLD and AVS compared with NAFLD and MAC, in patients with or without type 2 diabetes. This issue is of clinical significance, because the aortic and mitral valves are

different anatomically and are exposed to different intra-myocardial pressure gradients during the cardiac cycle.

Thus, the aim of this study was to examine whether NAFLD is associated with AVS and MAC (singly or in combination) in a large sample of patients with type 2 diabetes.

MATERIALS AND METHODS

Patients

We studied 247 white consecutive outpatients with type 2 diabetes, who regularly attended the diabetes clinics of the University of Verona and the "Sacro Cuore" Hospital of Negrar. Some data from a part of these patients (n=180) reporting associations between NAFLD and AVS was published previously [18]. For the present analyses, we excluded patients with: (1) a prior history of chronic heart failure, heart valve diseases or prosthetic heart valves, atrial fibrillation, cancer and overt nephropathy; and (2) a prior history of cirrhosis of any etiology or other known causes of chronic liver diseases, including viral hepatitis, hemochromatosis and excessive alcohol intake (defined as >20 g/day for women and >30 g/day of alcohol intake for men, respectively). All women were postmenopausal and did not take hormonal replacement therapy.

The local Ethics Committee 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 calculated by dividing weight in kilograms by height in meters squared. Waist circumference was measured at the level of the umbilicus. Blood pressure was measured with a standard mercury sphygmomanometer (at the right upper arm using an appropriate cuff size). Information on alcohol consumption, smoking status and current use of medications was obtained from all patients via interviews during medical examinations.

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155 Venous blood samples were drawn in the morning after an overnight fast. Serum liver enzymes,
156 creatinine (measured using a Jaffé rate-blanked and compensated assay) and other biochemical blood
157 measurements were determined using standard laboratory procedures (DAX 96; Bayer Diagnostics,
158 Milan, Italy). Most participants had serum liver enzymes within the reference ranges in our laboratory,
159 which for serum aminotransferases were 10 to 40 U/L for women and 10 to 50 U/L for men,
160 respectively. Low-density lipoprotein (LDL)-cholesterol was calculated using the Friedewald
161 equation. Hemoglobin A1c (HbA1c) was measured by an automated high-performance liquid
162 chromatography analyzer (HA-8140; Menarini Diagnostics, Florence, Italy); the upper limit of normal
163 for the laboratory was 5.6%. The glomerular filtration rate (eGFR) was estimated by the four-variable
164 Modification of Diet in Renal Disease study equation [19]. Albuminuria was measured by an immuno-
165 nephelometric method on a morning spot urine sample and expressed as the albumin-to-creatinine
166 ratio; abnormal albuminuria was defined as an albumin-to-creatinine ratio ≥30 mg/g creatinine.
167

168 Presence of IHD was defined as a documented history of myocardial infarction, angina or coronary
169 revascularization procedures. Presence of internal or common carotid artery stenoses was ascertained
170 by echo-Doppler scanning. In all participants, the presence of microvascular complications, such as
171 retinopathy (by fundoscopy after pupillary dilation), peripheral sensory neuropathy (by
172 biothesiometer) and nephropathy (by eGFR and albuminuria measurements) were also recorded.
173

174 **Echocardiography and Liver Ultrasonography**

175 A 12-lead standard resting electrocardiogram and a transthoracic echocardiographic Doppler
176 evaluation with spectral tissue Doppler analysis (Vivid 7, GE Vingmed, Horten, Norway) were
177 performed within ~1 month of liver ultrasonography in all patients by two experienced cardiologists,
178 who were blinded to the participants' details, including liver ultrasound data. Conventional
179 echocardiography was used to measure left ventricular (LV) diameters, wall thickness, and mass
180 according to standard criteria. LV end-diastolic and end-systolic volumes and ejection fraction at rest
181 were measured at the apical 4-chamber and 2-chamber views (by modified Simpson rule) [20]. Left

atrial maximal volume was measured at the end of LV systole from the apical 4-chamber and 2-chamber views (by modified Simpson rule) [20,21]. Pulsed-wave Doppler was used to measure trans-mitral peak early diastolic velocity (E), peak late diastolic velocity (A) and E-wave deceleration time (Dte). Pulsed-wave tissue Doppler echocardiography of the septal and lateral mitral annulus was also used to measure the early peak (e') and late (a') annular diastolic and systolic (s') tissue velocities [22,24].

Presence of AVS was defined according to international guidelines as either focal or diffuse calcification of the aortic leaflets without significant obstruction to left ventricular outflow, with a trans-aortic peak instantaneous velocity <2.6 m/s [2-9,20]. Presence of MAC was defined as an intense echocardiographic-producing structure with highly reflective characteristics that was located at the junction of the atrio-ventricular groove and the posterior or anterior mitral leaflet on the parasternal long-axis, short-axis, or apical 4-chamber view [6-9,20].

Liver ultrasonography was performed in all participants by two experienced radiologists, who were blinded to the participants' details, including echocardiographic data. Hepatic steatosis was diagnosed on the basis of characteristic ultrasonographic features, *i.e.,* evidence of diffuse hyper-echogenicity of the liver relative to the kidneys, ultrasound beam attenuation, and poor visualization of intra-hepatic vessel borders and diaphragm [25]. It is known that liver ultrasonography has a good sensitivity and specificity for detecting moderate and severe hepatic steatosis (~90-95%), but its sensitivity is reduced when the hepatic fat infiltration identified by liver biopsy is <30% [10,25]. Semi-quantitative ultrasonographic scoring for the degree of hepatic steatosis (mild, moderate or severe) was not available in this study. Grading of hepatic fat content using ultrasonography has been used in previous studies but remains somewhat subjective [25].

Statistical Analysis

Data are expressed as means±SD or proportions. We calculated a HVC score combining the presence of calcium at different valve sites as follows: HVC-0: absence of any calcification at the level of the

aortic and mitral valve; HVC-1 presence of either isolated AVS or isolated MAC; HVC-2: coexistence of AVS and MAC [6,9]. Differences in clinical/biochemical characteristics and echocardiographic parameters among patients stratified by the HVC score were tested with the one-way ANOVA for normally distributed variables and the Kruskal-Wallis test for non-normally distributed variables (*i.e.*, diabetes duration, liver enzymes and triglycerides). The X^2 test was used for categorical variables to study differences in proportions or percentages among the groups. Binary logistic regression analysis was also used to examine the association between NAFLD and the HVC score, and to identify the factors that were independently associated with the presence of AVS and/or MAC, which was included as the dependent variable (HVC-0 *vs.* combined HVC-1 and HVC-2). Four forced-entry logistic regression models were performed: an unadjusted model; a model adjusted for age and sex (model 1); a regression model further adjusted for waist circumference, smoking history, HbA1c, LDL-cholesterol, eGFR, diastolic blood pressure and current use of any hypoglycemic, lipid-lowering and anti-hypertensive drugs (model 2); and, finally, a regression model adjusted for the same variables included in model 2 *plus* the E/e' ratio (model 3). The covariates for multivariate regression models were chosen as potential confounding factors based on their significance in univariable regression analyses or based on their biological plausibility (*i.e.*, age, sex, waist circumference, smoking and LDL-cholesterol). Interaction terms were also generated between NAFLD and sex, age and waist circumference in terms of valvular calcification. None of these interaction terms was statistically significant in the fully adjusted regression models. P-values <0.05 were considered statistically significant.

RESULTS

Of the 247 patients included in the study, 175 (70.8%) patients met the clinical criteria for a diagnosis of NAFLD (*i.e.*, hepatic steatosis on ultrasonography among patients who did not have excessive alcohol consumption, viral hepatitis or other known causes of hepatic steatosis) and 72 patients did not. No patients had clinical, biochemical characteristics (including platelet count, liver enzymes,

albumin and prothrombin time) or ultrasonographic findings suggestive of cirrhosis or portal hypertension (coarse liver texture or splenomegaly).

Overall, 139 (56.3%) patients had no heart valve calcification (HVC-0), 65 (26.3%) patients had either isolated AVS or isolated MAC (HVC-1), of whom 42 patients had isolated AVS and 23 had isolated MAC. The remaining 43 (17.4%) patients had combined AVS and MAC (HVC-2). No patients had aortic stenosis (*i.e.*, defined as a trans-aortic peak instantaneous velocity ≥2.6 m/s), bicuspid aortic valve disease and mitral stenosis. In addition, a few patients had mild aortic/mitral regurgitation, but none of them had moderate or severe aortic and mitral regurgitation.

Table 1 shows the clinical and biochemical characteristics of patients with type 2 diabetes stratified by HVC scores. Diastolic blood pressure, HbA1c and serum gamma-glutamyltransferase levels increased, whereas eGFR levels decreased across HVC scores. Similarly, compared with those with HVC-0, patients with HVC-2 had a greater prevalence of chronic kidney disease (*i.e.*, eGFR <60 ml/min/1.73 m^2 and/or abnormal albuminuria), diabetic retinopathy and insulin treatment, and were more likely to be treated for dyslipidemia and hypertension (especially ACE-inhibitors, angiotensin receptor blockers or calcium-channel blockers). Notably, the prevalence of NAFLD markedly increased in patients with HVC-2 compared with either HVC-1 or HVC-0 (86.1% *vs.* 83.1% *vs.* 60.4%, respectively; p<0.001). Sex, age, BMI, waist circumference, duration of diabetes, systolic blood pressure, pulse pressure, smoking, fasting glucose levels, lipids, serum aminotransferases, presence of carotid artery stenoses, IHD and peripheral sensory neuropathy, and the proportion using beta-blockers and diuretics did not differ significantly among the three groups of patients.

Table 2 shows the main echocardiographic parameters of patients grouped according to their HVC score. Left atrial volume index and E/e' ratio (*i.e.*, an index of LV diastolic filling pressure) increased progressively across the HVC score. No significant differences were observed in LV volumes, LV-ejection fraction, E/A ratio, Dte, tau, IVRT, SAC and SVR index among the three groups of patients.

Figure 1 shows the prevalence of different levels of HVC score among patients with and without NAFLD. Of note, approximately 50% of patients with NAFLD had any valvular calcification at the level of aortic and mitral valves compared with ~20% of patients who did not have NAFLD.

Table 3 shows the effect of the adjustment for potential confounders on the association between NAFLD and the presence of AVS, MAC or both. In univariable regression analysis, NAFLD was associated with a 3.5-fold increased rate of valvular calcification (unadjusted-odds ratio 3.51, 95% CI 1.89-6.51). Adjustments for age, sex, waist circumference, smoking, HbA1c, LDL-cholesterol, eGFR, diastolic blood pressure and use of medications (model 2) did not appreciably weaken this association. Further adjustment for the E/e' ratio (model 3) did not attenuate the significant association between NAFLD and valvular calcification. Almost identical results were found when patients with a prior history of IHD (n=25) were excluded from analysis. Interestingly, as also shown in **Table 3**, other independent predictors of valvular calcification were higher HbA1c and lower eGFR levels. Results remained unchanged even when we additionally adjusted for LV mass index and left atrial volume index (data not shown).

Table 4 shows the effect of the adjustment for the same set of covariates we used in Table 3 on the relationship between NAFLD and AVS or MAC, separately. Consistent with the other results, NAFLD was associated with an approximately 2.5-fold to 3-fold higher risk of having AVS or MAC after adjustment for potential confounders.

DISCUSSION

The major finding of our study is that the prevalence of NAFLD was highest in patients with coexisting AVS and MAC (*i.e.*, HVC-2), intermediate in those with either isolated AVS or isolated MAC (HVC-1), and lowest in those without valvular calcification (HVC-0). Notably and most importantly, the association between NAFLD and HVC score was independent of multiple

294 cardiovascular risk factors and potential confounders, including diabetes-related variables, eGFR, use
295 of medications, and echocardiographic parameters. Of note, other independent predictors of valvular
296 calcification in these patients were lower eGFR values and poor glycemic control.
297
298 To date, very little information is currently available regarding the association between NAFLD and
299 presence of calcification of the aortic and mitral valves, especially in people with type 2 diabetes, and
300 it is uncertain whether similar associations exist between both NAFLD and AVS and NAFLD and
301 MAC. It is known that the process of calcification of the aortic and mitral valves is associated with
302 established risk factors for atherosclerosis, and that valvular calcification shares many of its
303 histological and molecular characteristics with atherosclerosis [1,2]. It is also known that the presence
304 of diabetes is predictive of poor prognosis in native valve disease and of faster degeneration of
305 implanted bio-prosthetic aortic valves [26,27]. Similarly, increasing evidence indicates that valvular
306 calcification independently predicts adverse cardiovascular outcomes among patients with end-stage
307 kidney disease [28,29]. However, it is important to note that the Framingham Heart Study
308 investigators have recently shown that decreased kidney function is significantly associated with the
309 presence of valvular calcification before the onset of end-stage kidney disease in middle-aged
310 individuals [30]. Similar findings were also noted by the investigators of the Cardiovascular Health
311 Study in a community-based cohort of older adults [31] and by the investigators of the Multi-Ethnic
312 Study of Atherosclerosis in a racially and ethnically diverse middle-aged population [32].
313
314 Our findings expand to diabetic patients the recent observations by Markus *et al.* who showed that
315 fatty liver on ultrasonography is associated with an increased prevalence of AVS, independently of
316 multiple cardiovascular and metabolic risk factors, in a cohort of 2,212 German men and women (aged
317 45–81 years) [17]. However, these authors did not analyze the association between fatty liver and
318 MAC, or between fatty liver and combined AVS and MAC. In addition, they did not perform separate
319 statistical analyses stratifying by diabetes status. More recently, in a sample of 180 patients with type 2
320 diabetes, we found that NAFLD and AVS were strictly inter-related after adjustment for major
321 confounders [18]. In that study, however, we did not observe any significant association between

NAFLD and the presence of MAC (unadjusted-OR 1.32, 95% CI 0.6-2.9, p=0.52) [18]. Although it is possible that our failure to observe an association between NAFLD and MAC may have been due to the relatively small sample size and inadequate power to detect an association, it was also plausible that different associations exist between NAFLD and MAC as exist for NAFLD and AVS.

To our knowledge, this is the largest cross-sectional study reporting a significant association between NAFLD and MAC, and between NAFLD and combined AVS and MAC in a sample of type 2 diabetic individuals with preserved LV systolic function.

Large prospective studies reported that AVS and MAC are independently associated with an increased risk of future cardiovascular events [3-8]. In addition, the presence of MAC, but not of AVS, is independently associated with an increased risk of incident atrial fibrillation (AF) and improves AF risk prediction [33]. This suggests that the presence of MAC may not only mark increased cardiovascular risk, but in some cases may increase the risk of incident AF. Overall, therefore, our findings suggest that calcification of the aortic and mitral valves may represent a further link underpinning the increased risk of developing both cardiovascular disease and AF observed in patients with NAFLD. These findings further highlight the clinical importance of evaluating risk of cardiovascular disease among patients with NAFLD [10,14-16]. Our findings also suggest that the echocardiographic detection of MAC and/or AVS in type 2 diabetic patients with NAFLD may identify a subgroup of patients, who are at higher risk of developing cardiovascular disease and AF than had previously been appreciated. Another clinical implication of our findings is that once diagnosis of echocardiographic AVS or MAC has been made, an aggressive management of modifiable cardiovascular risk factors is essential also to reduce the risk of progression of these valvular diseases. Future studies are needed to examine whether a change in NAFLD status (either development of new fatty liver, progression to cirrhosis, or resolution of existing fatty liver) over time can modify the risk of progression or regression of AVS or MAC.

To date, the underlying mechanisms responsible for the observed associations between NAFLD and AVS or MAC are incompletely understood. The most obvious explanation for our findings is that the association between NAFLD and calcification of the aortic and mitral valves is simply a marker of coexisting cardiovascular risk factors, including poor glycemic control, decreased eGFR, hypertension, dyslipidemia, smoking and prior IHD. However, in our analyses, the association between NAFLD and valvular calcification persisted after adjustment for these potential confounders. Furthermore, the association between NAFLD and valvular calcification was essentially unaltered after excluding patients with IHD. This suggests that additional mechanisms, beyond classic cardiovascular and metabolic risk factors, could, in part, be responsible for the observed association. Accumulating clinical and experimental evidence indicates that NAFLD may exacerbate hepatic/peripheral insulin resistance, induces atherogenic dyslipidemia and releases a variety of pro-atherogenic mediators (*e.g.*, C-reactive protein, interleukin-6, tumor growth factor-beta, osteopontin, plasminogen activator inhibitor-1 and other pro-coagulant and pro-fibrogenic mediators) that play important roles in the pathophysiology of vascular and valvular calcification [10,11,14,16,34-37]. Preliminary evidence also suggests that the coexistence of obesity-related increases in fat accumulation in the myocardium may additionally exert local adverse effects that result in myocardial and valvular alterations [16]. Mahmod *et al.* showed that myocardial steatosis, as detected by proton magnetic resonance spectroscopy, was common in patients with severe aortic stenosis, and that myocardial steatosis was significantly associated with LV systolic dysfunction among these patients [38]. However, in a recent elegant study assessing the effect of different ectopic fat depots on LV function in non-diabetic men with NAFLD, Granér *et al.* reported that only intra-hepatic triglyceride content and visceral adipose tissue were independent predictors of LV diastolic function, whereas myocardial steatosis, epicardial and pericardial fat were not significantly associated with diastolic function measures [39]. This further supports the possibility that the association between NAFLD and valvular calcification may be because of toxic systemic effects. However, further studies are needed in order to improve understanding of these issues.

376 Collectively, although the potential role of NAFLD in the pathophysiology of vascular and valvular
377 calcification requires further confirmation in larger well-designed studies, we believe that this is a
378 promising field of research to explore, and that the pathways that involve the contribution of NAFLD
379 *per se* to chronic inflammation, hypercoagulation and activation of Wnt signaling pathway might
380 provide a potential therapeutic target for the treatment and prevention of vascular and valvular
381 calcification in diabetic people with NAFLD [1,36,37,40-42]. In line with our data, it is also possible
382 to hypothesize that another underlying mechanism by which NAFLD may promote vascular and
383 valvular calcification could be partly mediated by the development of kidney damage [43,44].
384
385 Our study has several important strengths, including the large sample size, the completeness of the
386 dataset, and the ability to adjust for multiple risk factors and potential confounders, including diabetes-
387 related variables, kidney function parameters and use of medications. In addition, our patients were
388 free of cirrhosis; we believe that the inclusion of patients with such complication would have
389 confounded the interpretation of data.
390
391 Despite the comprehensive nature of the data set, there are some important limitations to our study.
392 Firstly, the cross-sectional design of the study limits our ability to establish the temporality of the
393 observed associations. Secondly, the diagnosis of NAFLD was based on ultrasound imaging and the
394 exclusion of other known aetiological factors of chronic liver diseases, but was not confirmed by liver
395 biopsy. Although some non-differential misclassification of NAFLD on the basis of ultrasonography is
396 likely (*i.e.*, some of our diabetic control patients could have underlying NAFLD despite fairly normal
397 serum liver enzymes and negative ultrasonography examination), this limitation would serve to
398 attenuate the magnitude of our effect measures toward null; thus, our data can probably be considered
399 conservative estimates of the relationship between NAFLD and valvular calcification. Finally, because
400 our sample comprised white type 2 diabetic individuals who were followed at outpatient clinics, our
401 results may not necessarily be generalizable to other non-white diabetic populations.
402

In conclusion, these results provide further information about the relationship between NAFLD and heart valve calcification, demonstrating that NAFLD is strongly and independently associated with AVS and MAC (singly or in combination) in patients with type 2 diabetes. Future studies are needed to elucidate whether NAFLD may predict the development and progression of valvular calcification.

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REFERENCES

[1] Otto CM, Prendergast B. Aortic-valve stenosis--from patients at risk to severe valve obstruction. N Engl J Med 2014;371:744-756.

[2] Rajamannan NM, Evans FJ, Aikawa E, Grande-Allen KJ, Demer LL, Heistad DD, Simmons CA, Masters KS, Mathieu P, O'Brien KD, Schoen FJ, Towler DA, Yoganathan AP, Otto CM. Calcific aortic valve disease: not simply a degenerative process: A review and agenda for research from the National Heart and Lung and Blood Institute Aortic Stenosis Working Group. Executive summary: Calcific aortic valve disease-2011 update. Circulation 2011;124:1783-1791.

[3] Otto CM, Lind BK, Kitzman DW, Gersh BJ, Siscovick DS. Association of aortic-valve sclerosis with cardiovascular mortality and morbidity in the elderly. N Engl J Med 1999;341:142-147.

433 [4] Barasch E, Gottdiener JS, Marino Larsen EK, Chaves PH, Newman AB. Cardiovascular morbidity
434 and mortality in community-dwelling elderly individuals with calcification of the fibrous skeleton of
435 the base of the heart and aortosclerosis (The Cardiovascular Health Study). Am J Cardiol
436 2006;97:1281-1286.
437 [5] Nasir K, Katz R, Takasu J, Shavelle DM, Detrano R, Lima JA, Blumenthal RS, O'Brien K, Budoff
438 MJ. Ethnic differences between extra-coronary measures on cardiac computed tomography: multi-
439 ethnic study of atherosclerosis (MESA). Atherosclerosis 2008;198:104-114.
440 [6] Völzke H, Haring R, Lorbeer R, Wallaschofski H, Reffelmann T, Empen K, Rettig R, John U,
441 Felix SB, Dörr M. Heart valve sclerosis predicts all-cause and cardiovascular mortality.
442 Atherosclerosis 2010;209:606-610.
443 [7] Fox CS, Vasan RS, Parise H, Levy D, O'Donnell CJ, D'Agostino RB, Benjamin EJ; Framingham
444 Heart Study. Mitral annular calcification predicts cardiovascular morbidity and mortality: the
445 Framingham Heart Study. Circulation 2003;107:1492-1496.
446 [8] Kohsaka S, Jin Z, Rundek T, Boden-Albala B, Homma S, Sacco RL, Di Tullio MR. Impact of
447 mitral annular calcification on cardiovascular events in a multiethnic community: the Northern
448 Manhattan Study. JACC Cardiovasc Imaging 2008;1:617-623.
449 [9] Rossi A, Targher G, Zoppini G, Cicoira MA, Bonapace S, Negri C, Stoico V, Faggiano P,
450 Vassanelli C, Bonora E. Aortic and mitral annular calcifications are predictive of all-cause and
451 cardiovascular mortality in patients with type 2 diabetes. Diabetes Care 2012;35:1781-1786.
452 [10] Anstee QM, Targher G, Day CP. Progression of NAFLD to diabetes mellitus, cardiovascular
453 disease or cirrhosis. Nat Rev Gastroenterol Hepatol 2013;10:330-344.
454 [11] Targher G, Byrne CD. Clinical review: nonalcoholic fatty liver disease: a novel cardiometabolic
455 risk factor for type 2 diabetes and its complications. J Clin Endocrinol Metab 2013;98:483-495.
456 [12] Targher G, Bertolini L, Padovani R, Rodella S, Tessari R, Zenari L, Day C, Arcaro G. Prevalence
457 of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2
458 diabetic patients. Diabetes Care 2007;30:1212-1218.
459 [13] Leite NC, Salles GF, Araujo AL, Villela-Nogueira CA, Cardoso CR. Prevalence and associated
460 factors of non-alcoholic fatty liver disease in patients with type 2 diabetes mellitus. Liver Int
461 2009;29:113-119.
462 [14] Targher G, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty
463 liver disease. N Engl J Med 2010;363:1341-1350.
464 [15] Armstrong MJ, Adams LA, Canbay A, Syn WK. Extra-hepatic complications of nonalcoholic
465 fatty liver disease. Hepatology 2014;59:1174-1197.
466 [16] Ballestri S, Lonardo A, Bonapace S, Byrne CD, Loria P, Targher G. Risk of cardiovascular,
467 cardiac and arrhythmic complications in patients with non-alcoholic fatty liver disease. World J
468 Gastroenterol 2014;20:1724-1745.

469 [17] Markus MR, Baumeister SE, Stritzke J, Dorr M, Wallaschofski H, Volke H, Lieb W. Hepatic
470 steatosis is associated with aortic valve sclerosis in the general population. The Study of Health in
471 Pomerania (SHIP). Arterioscler Thromb Vasc Biol 2013;33:1690-1695.
472 [18] Bonapace S, Valbusa F, Bertolini l, Pichiri I, Mantovani A, Rossi A, Zenari L, Barbieri E,
473 Targher G. Nonalcoholic fatty liver disease is associated with aortic valve sclerosis in patients with
474 type 2 diabetes mellitus. Plos One 2014;9:e88371.
475 [19] Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to
476 estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of
477 Diet in Renal Disease Study Group. Ann Intern Med 1999;130:461-470.
478 [20] Lang RM, Bierig M, Devereux RB, Flachskampf FA, Foster E, Pellikka PA, Picard MH, Roman
479 MJ, Seward J, Shanewise JS, Solomon SD, Spencer KT, Sutton MS, Stewart WJ; American Society of
480 Echocardiography's Nomenclature and Standards Committee; Task Force on Chamber Quantification;
481 American College of Cardiology Echocardiography Committee; American Heart Association;
482 European Association of Echocardiography, European Society of Cardiology. Recommendations for
483 chamber quantification. Eur J Echocardiogr 2006;7:79-108.
484 [21] Nagueh SF, Appleton CP, Gillebert TC, Marino PN, Oh JK, Smiseth OA, Waggoner AD,
485 Flachskampf FA, Pellikka PA, Evangelisa A. Recommendations for the evaluation of left ventricular
486 diastolic function by echocardiography. J Am Soc Echocardiogr 2009;22:107-133.
487 [22] Nagueh SF, Middleton KJ, Kopelen HA, Zoghbi WA, Quiñones MA. Doppler tissue imaging: a
488 noninvasive technique for evaluation of left ventricular relaxation and estimation of filling pressures. J
489 Am Coll Cardiol 1997;30:1527-1533.
490 [23] Bonapace S, Targher G, Molon G, Rossi A, Costa A, Zenari L, Bertolini L, Cian D, Lanzoni L,
491 Barbieri E. Relationship between early diastolic dysfunction and abnormal microvolt T-wave alternans
492 in patients with type 2 diabetes. Circ Cardiovasc Imaging 2011;4:408-414.
493 [24] Bonapace S, Perseghin G, Molon G, Canali G, Bertolini L, Zoppini G, Barbieri E, Targher G.
494 Nonalcoholic fatty liver disease is associated with left ventricular diastolic dysfunction in patients with
495 type 2 diabetes. Diabetes Care 2012;35:389-395.
496 [25] Hernaez R, Lazo M, Bonekamp S, Kamel I, Brancati FL, Guallar E, Clark JM. Diagnostic
497 accuracy and reliability of ultrasonography for the detection of fatty liver: a meta-analysis. Hepatology
498 2011;54:1082-1090.
499 [26] Katz R, Budoff MJ, Takasu J, Shavelle DM, Bertoni A, Blumenthal RS, Ouyang P, Wong ND,
500 O'Brien KD. Relationship of metabolic syndrome with incident aortic valve calcium and aortic valve
501 calcium progression: the Multi-Ethnic Study of Atherosclerosis (MESA). Diabetes 2009;58:813-819.
502 [27] Lorusso R, Gelsomino S, Lucà F, De Cicco G, Billè G, Carella R, Villa E, Troise G, Viganò M,
503 Banfi C, Gazzaruso C, Gagliardotto P, Menicanti L, Formica F, Paolini G, Benussi S, Alfieri O,
504 Pastore M, Ferrarese S, Mariscalco G, Di Credico G, Leva C, Russo C, Cannata A, Trevisan R, Livi
505 U, Scrofani R, Antona C, Sala A, Gensini GF, Maessen J, Giustina A. Type 2 diabetes mellitus is

associated with faster degeneration of bioprosthetic valve: results from a propensity score-matched Italian multicenter study. Circulation 2012;125:604-614.

[28] Maher ER, Young G, Smyth-Walsh B, Pugh S, Curtis JR. Aortic and mitral valve calcification in patients with end-stage renal disease. Lancet 1987;2:875-877.

[29] Wang AY, Wang M, Woo J, Lam CW, Li PK, Lui SF, Sanderson JE. Cardiac valve calcification as an important predictor for all-cause mortality and cardiovascular mortality in long-term peritoneal dialysis patients: A prospective study. J Am Soc Nephrol 2003;14:159-168.

[30] Fox CS, Larson MG, Vasan RS, Guo CY, Parise H, Levy D, Leip EP, O'Donnell CJ, D'Agostino RB Sr, Benjamin EJ. Cross-sectional association of kidney function with valvular and annular calcification: the Framingham heart study. J Am Soc Nephrol 2006;17:521-527.

[31] Asselbergs FW1, Mozaffarian D, Katz R, Kestenbaum B, Fried LF, Gottdiener JS, Shlipak MG, Siscovick DS. Association of renal function with cardiac calcifications in older adults: the cardiovascular health study. Nephrol Dial Transplant 2009;24:834-840.

[32] Ix JH, Shlipak MG, Katz R, Budoff MJ, Shavelle DM, Probstfield JL, Takasu J, Detrano R, O'Brien KD. Kidney function and aortic valve and mitral annular calcification in the Multi-Ethnic Study of Atherosclerosis (MESA). Am J Kidney Dis 2007;50:412-420.

[33] O'Neal WT, Efird JT, Nazarian S, Alonso A, Heckbert SR, Soliman EZ. Mitral annular calcification and incident atrial fibrillation in the Multi-Ethnic Study of Atherosclerosis. Europace 2015;17:358-363.

[34] Polyzos SA, Kountouras J, Mantzoros CS. Leptin in nonalcoholic fatty liver disease: a narrative review. Metabolism 2015;64:60-78.

[35] Liu J, Xu Y, Hu Y, Wang G. The role of fibroblast growth factor 21 in the pathogenesis of non-alcoholic fatty liver disease and implications for therapy. Metabolism 2015;64:380-390.

[36] Targher G, Byrne CD. Diagnosis and management of nonalcoholic fatty liver disease and its hemostatic/thrombotic and vascular complications. Semin Thromb Hemost 2013;39:214-228.

[37] Virmani R, Joner M, Sakakura K. Recent highlights of ATVB: calcification. Arterioscler Thromb Vasc Biol 2014;34:1329-1332.

[38] Mahmod M, Bull S, Suttie JJ, Pal N, Holloway C, Dass S, Myerson SG, Schneider JE, De Silva R, Petrou M, Sayeed R, Westaby S, Clelland C, Francis JM, Ashrafian H, Karamitsos TD, Neubauer S. Myocardial steatosis and left ventricular contractile dysfunction in patients with severe aortic stenosis. Circ Cardiovasc Imaging 2013;6:808-816.

[39] Granér M, Nyman K, Siren R, Pentikäinen MO, Lundbom J, Hakkarainen A, Lauerma K, Lundbom N, Nieminen MS, Taskinen MR. Ectopic fat depots and left ventricular function in nondiabetic men with nonalcoholic fatty liver disease. Circ Cardiovasc Imaging 2014 Dec 30; doi: 10.1161/CIRCIMAGING.114.001979.

[40] Maruotti N, Corrado A, Neve A, Cantatore FP. Systemic effects of Wnt signaling. J Cell Physiol 2013;228:1428-1432.

543 [41] Fox CS, Guo CY, Larson MG, Vasan RS, Parise H, O'Donnell CJ, D'Agostino RB Sr, Keaney JF
544 Jr, Benjamin EJ. Relations of inflammation and novel risk factors to valvular calcification. Am J
545 Cardiol 2006;97:1502-1505.
546 [42] Natorska J, Undas A. Blood coagulation and fibrinolysis in aortic valve stenosis: links with
547 inflammation and calcification. Thromb Haemost 2015 Mar 26;114(1) [Epub ahead of print].
548 [43] Chang Y, Ryu S, Sung E, Woo HY, Oh E, Cha K, Jung E, Kim WS. Nonalcoholic fatty liver
549 disease predicts chronic kidney disease in non-hypertensive and nondiabetic Korean men. Metabolism
550 2008;57:569-576.
551 [44] Targher G, Chonchol MB, Byrne CD. CKD and nonalcoholic fatty liver disease. Am J Kidney
552 Dis 2014;64:638-652.
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558 **Table 1.** Clinical and biochemical characteristics of patients with type 2 diabetes stratified by status of heart
559 valve calcification (HVC).
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	HVC-0 (n=139)	*HVC-1 (n=65)*	*HVC-2 (n=43)*	*P value*
Sex (male/female)	105/34	45/20	29/14	0.46
Age (years)	67.6±7	69.1±8	69.7±5	0.12
Body weight (kg)	82.1±14	82.6±14	81.6±15	0.93
Body mass index (kg/m^2)	28.8±5	28.6±4	29.1±5	0.88
Waist circumference (cm)	97.8±13	97.1±16	98.6±16	0.43
Diabetes duration (years)	13±10	14±10	14±9	0.49
Systolic blood pressure (mmHg)	142±17	145±15	141±13	0.34
Diastolic blood pressure (mmHg)	77±8	81±9	79±8	<0.05
Pulse pressure (mmHg)	63±14	64±14	64±12	0.98
Mean arterial pressure (mmHg)	98±10	101±9	99±10	0.21
Heart rate (bpm)	74±11	75±12	73±8	0.79
Smoking history (%)	30.2	29.2	32.6	0.80
Fasting glucose (mmol/l)	8.1±2.3	7.8±2.1	8.1±2.5	0.55
Hemoglobin A1c (%)	7.1±0.9	7.4±1.6	7.6±1.4	<0.01
Total cholesterol (mmol/l)	4.44±1.0	4.50±1.1	4.33±1.0	0.50
HDL-cholesterol (mmol/l)	1.26±0.3	1.27±0.3	1.22±0.2	0.59
LDL-cholesterol (mmol/l)	2.57±0.8	2.54±0.8	2.41±0.8	0.47
Triglycerides (mmol/l)	1.54±0.7	1.51±0.8	1.76±0.9	0.18
eGFR (ml/min/1.73 m^2)	84.1±19	83.9±18	66.5±24	<0.001
AST (U/l)	20±5	23±6	26±7	0.19
ALT (U/l)	23±5	26±7	30±9	0.08
GGT (U/l)	24±7	33±12	36±14	<0.05
Hypertension (%)	77.4	81.5	91.0	0.08
eGFR <60 ml/min/1.73 m^2 (%)	7.9	7.7	30.2	<0.001
Abnormal albuminuria (%)	12.2	32.3	25.7	<0.05

Diabetic retinopathy, any degree (%)	14.4	15.3	34.9	<0.005
Diabetic sensory neuropathy (%)	13.0	13.8	13.8	0.64
Carotid artery stenosis ≥50 % (%)	17.3	17.0	25.6	0.43
History of IHD (%)	10.0	9.2	11.6	0.92
Oral hypoglycemic drug users (%)	79.1	86.2	62.8	<0.05
Insulin users (%)	36.7	35.3	46.5	<0.05
ACE-inhibitor/ARB users (%)	73.4	72.3	90.7	<0.05
Calcium-channel blocker users (%)	32.4	23.1	44.2	0.07
Diuretic users (%)	40.3	35.4	41.9	0.74
Beta-blocker users (%)	21.6	21.5	18.6	0.91
Statin users (%)	76.2	72.3	90.7	<0.05
NAFLD (%)	60.4	83.1	86.1	<0.001
AVS (%)	0	64.6	100	ND
MAC (%)	0	35.4	100	ND

Sample size, n=247. Data are expressed as means±SD or percentages. ND, not determined.

Hypertension was defined as blood pressure ≥140/90 mmHg and/or use of any anti-hypertensive drugs.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ARB, angiotensin receptor blockers; AVS, aortic valve sclerosis; eGFR, estimated glomerular filtration rate; GGT, gamma-glutamyltransferase; HVC, heart valve calcification; IHD, ischemic heart disease; MAC, mitral annular calcification.

575 **Table 2**. Main echocardiographic characteristics of patients with type 2 diabetes stratified by status of heart
576 valve calcification (HVC).

	HVC-0 (n=139)	*HVC-1 (n=65)*	*HVC-2 (n=43)*	*P value*
LV end-diastolic volume (ml)	105±22	111±26	109±36	0.23
LV end-systolic volume (ml)	38±14	42± 5	43±24	0.16
LV ejection fraction (%)	64±7	62±7	62±8	0.21
LV mass index (g/m^2)	107±27	106±18	111±30	0.62
LA volume index (ml/m^2)	29±9	33±9	35±15	<0.01
E/A wave ratio	0.75±0.2	0.77±0.2	0.77±0.2	0.54
Dte (ms)	259±66	241±61	249±56	0.17
E/e' ratio	8.30±2.3	9.52±2.7	9.97±3.0	<0.001
IVRT (ms)	85±15	85±17	88±15	0.54
Tau (ms)	45±14	47±12	51±14	0.16
SAC (mmHg/ml)	1.10±0.3	1.15±0.4	1.11±0.4	0.64
SVR index ($dyne/s/cm^5$)	2497±792	2504±819	2691±847	0.25

577 Sample size, n=247. Data are expressed as means±SD.

578 HVC, heart valve calcification; IVRT, isovolumetric relaxation time; LA, left atrial; LV, left ventricular;
579 SAC, systemic arterial compliance; SVR, systemic vascular resistance; Tau, time constant of isovolumic
580 relaxation.

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Table 3. Independent predictors of the presence of valvular calcification (defined as presence of AVS, MAC or both) in patients with type 2 diabetes.

Logistic Regression Models	**Odds ratio(s)**	**95% CI**	***P* value**
NAFLD (yes *vs.* no)			
Unadjusted model	3.51	1.89-6.51	<0.001
Adjusted model 1	3.29	1.76-6.16	<0.001
Adjusted model 2	3.88	1.69-8.94	<0.001
Adjusted model 3	2.70	1.23-7.38	<0.01
After excluding patients (*n*=25) with a prior history of ischemic heart disease			
Unadjusted model	3.77	1.95-7.26	<0.001
Adjusted model 1	3.55	1.83-6.90	<0.001
Adjusted model 2	4.12	2.01-9.02	<0.001
Adjusted model 3	3.48	1.44-8.54	<0.005
Other independent predictors of valvular calcifications in adjusted model 2			
HbA1c (%)	1.32	1.05-1.69	<0.05
eGFR (ml/min/1.73 m^2)	0.98	0.97-0.99	<0.005

Sample size, *n*=247 (except for the regression model 3 in which E/e' ratio measurements were available in 238 patients).

Data are expressed as odds ratios±95% confidence intervals (CI) as assessed by either univariable (unadjusted) or multivariable logistic regression analyses.

Other covariates included in multivariable regression models, along with NAFLD, were as follows: **model 1**: age and sex; **model 2**: age, sex, waist circumference, smoking history, HbA1c, LDL-cholesterol, eGFR, diastolic blood pressure, use of any hypoglycemic, lipid-lowering and anti-hypertensive drugs; **model 3**: adjustment for the same variables included in model 2 *plus* E/e' ratio.

Table 4. Independent predictors of the presence of either aortic valve stenosis (AVS) or mitral annulus calcification (MAC) in patients with type 2 diabetes.

Logistic Regression Models for AVS	**Odds ratio(s)**	**95% CI**	***P* value**
NAFLD (yes *vs.* no)			
Unadjusted model	3.58	1.80-7.12	<0.001
Adjusted model 1	3.35	1.67-6.75	<0.001
Adjusted model 2	3.82	1.78-8.14	<0.001
Adjusted model 3	2.65	1.07-6.31	=0.035
Logistic Regression Models for MAC	**Odds ratio(s)**	**95% CI**	***P* value**
NAFLD (yes *vs.* no)			
Unadjusted model	2.54	1.24-5.21	<0.01
Adjusted model 1	2.42	1.18-4.99	<0.01
Adjusted model 2	3.06	1.35-6.98	<0.005
Adjusted model 3	3.01	1.12-7.99	=0.026

Sample size, *n*=247 (except for the regression model 3 in which E/e' ratio measurements were available in 238 patients).

Data are expressed as odds ratios±95% confidence intervals (CI) as assessed by either univariable (unadjusted) or multivariable logistic regression analyses.

Other covariates included in multivariable regression models, along with NAFLD, were as follows: **model 1**: age and sex; **model 2**: age, sex, waist circumference, smoking history, HbA1c, LDL-cholesterol, eGFR, diastolic blood pressure, use of hypoglycemic, lipid-lowering and anti-hypertensive drugs; **model 3**: adjustment for the same variables included in model 2 *plus* the E/e' ratio.

FIGURE LEGENDS

Figure 1. Prevalence of HVC score in patients with type 2 diabetes stratified by NAFLD status. *P*-value <0.001 for the difference by the X^2 test.