Valvular Heart Disease Stages Among Older Adults And The Proteomic Markers of Aortic Stenosis: The Atherosclerosis Risk in Communities Study

Valvular Heart Disease Stages Among Older Adults

And The Proteomic Markers of Aortic Stenosis:

The Atherosclerosis Risk in Communities Study

By

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Overview:

Valvular heart disease (VHD) includes stenosis and regurgitation of the mitral and aortic valves (AV). The prevalence of moderate to severe VHD is ~2.5% in the general population, with aortic stenosis (AS) the most common moderate to severe valvular heart disease in the United States. The prevalence of less than severe VHD is more common. (1,2) VHD prevalence increases with advancing age, and the burden of VHD is expected to increase, as the US population ages.(3) The American College of Cardiology /American Heart Association (ACC/AHA) introduced the framework of VHD Stages to emphasize its progressive nature and potential opportunities for prevention.(4) However, little is known regarding the community-based prevalence, prognostic relevance, and progression of ACC/AHA VHD Stages, particularly those capturing non-severe VHD (i.e., Stage A and B). Furthermore, despite the progressive nature of VHD, biomarkers to identify persons at risk and interventions to prevent VHD progression are limited. The available biomarkers for its most common lesion (i.e., AS) – troponin and NT-proBNP – are related to sequelae of AS on the left ventricle as opposed to underlying mechanisms driving disease progression.(4)

In this work, we estimated the prevalence of VHD stages, their prognostic relevance for incident cardiovascular diseases, and their progression over 6 years in late-life. In order to enable preventive tools, we use high-throughput proteomics to discover potential biomarkers and molecular pathways related to the progression of AS.

Manuscript 1:

Stages of Valvular Heart Disease Among Older Adults in the Community: The Atherosclerosis Risk in Communities Study

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Abstract

Importance
Limited data exist regarding American College of Cardiology/American Heart Association (ACC/AHA) valvular heart disease (VHD) stage prevalence, progression, and association with incident cardiovascular diseases in late life.

Objective
Quantify VHD Stage prevalence and progression over six years in late-life and determine the independent association of VHD stage with incident cardiovascular diseases.

Design, Setting, and Participants
Cross-sectional, longitudinal, and time-to-event analyses using the data from participants in the Atherosclerosis Risk in Communities (ARIC) prospective community-based cohort study who underwent protocol echocardiography.

Exposures
VHD stages at ARIC Visits 5 (2011-2013) and 7 (2018-2019) defined based on ACC/AHA guidelines.

Main Outcomes and Measures
Incident adjudicated death, heart failure (HF), coronary heart disease (CHD), stroke, and atrial fibrillation (AF); and longitudinal changes in VHD prevalence over approximately 6 years. Cox proportional hazard models adjusted for age, sex, race, hypertension, diabetes, prior myocardial infarction, HF, body mass index, study center, and systolic blood pressure at Visit 5. Longitudinal changes in VHD stages were estimated using inverse probability of attrition weights (IPAW) to account participant attrition.

Results
Among 6,118 ARIC participants, mean ±SD age was 76±5 years, 42% were male, and 22% reported Black race. Stage A VHD was present in 39%, Stage B in 17%, and Stage C/D in 1.1%, while 0.7% had previously undergone valve replacement or repair. A graded association was observed between Stage A, B, and C/D VHD and risk of all-cause mortality, incident HF, incident AF, and incident CHD, but not incident stroke. Similar findings were observed for stages of each valvular lesion individually. During the 6.6 [IQR, 6.1-7.0] years between Visit 5 and Visit 7 (mean age 81±4 years), the prevalence of freedom from VHD stage decreased from 43% to 24%, while the prevalence of Stage C/D VHD increased from 1% to 7% respectively.

Conclusions and Relevance
Subclinical VHD is common in older adults, with 39% at risk (Stage A) and 17% with progressive VHD (Stage B), and is independently associated with risk of incident cardiovascular events. VHD stages progress over six years in late-life, with a several-fold increase in prevalence of severe VHD (Stage C/D), highlighting the public health importance of interventions to mitigate VHD progression.

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Dr. AM Shah had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Introduction

Valvular heart disease (VHD) is associated with significant morbidity and mortality and demonstrates a marked increase in prevalence with advancing age. While the prevalence of moderate to severe VHD is estimated at 2.5% in the general population, this estimate increases to 13.2% among those >75 years old.(1) Lesser degrees of valvular disease are even more common, occurring in approximately 51% of community-dwelling persons ≥65 years of age.(2) The burden of VHD is expected to grow substantially as the population ages, with persons >65 years old anticipated to account for 20% of the US population by 2030.(3)

VHD is progressive, beginning with structural alterations in valve morphology, moving through increasing degrees of valvular dysfunction (stenosis or regurgitation), and ultimately culminating in severe symptomatic disease. In 2014, the American College of Cardiology /American Heart Association (ACC/AHA) adopted the conceptual framework of VHD stages to emphasize its progressive nature.(4, 5) This schema defines VHD stages as: Stage A – at risk for valve dysfunction; Stage B – progressive valvular dysfunction; Stage C – severe asymptomatic valve dysfunction; and Stage D – severe symptomatic valve dysfunction. However, to our knowledge, no community-based estimates exist for the prevalence of VHD stages, or their progression over time, particularly in late-life when the burden of VHD is greatest. We aimed to (1) define the distribution of VHD stages; (2) determine the prognostic relevance of VHD stages for incident cardiovascular disease (CVD); and (3) characterize the progression in VHD stages over approximately 6 years among older adults in the community.

Methods

Study Population

The Atherosclerosis Risk in Communities (ARIC) study is a prospective epidemiologic cohort study, the design and methods of which have been previously described.(6) Between 1987 and 1989, 15,792 middle-aged subjects were enrolled in 4 communities in the United States: Forsyth County, NC, Jackson, MS, suburban Minneapolis, MN, and Washington County, MD. Of the 10,742 alive at the time of Visit 5 (2011 to 2013), 6,118 participants attended and underwent echocardiography with adequate images for assessment of VHD. Of these, 4,895 were alive at the time of Visit 7 (2018 to 2019), 2,896 of whom attended and underwent protocol echocardiography **(Figure 1)**.

Echocardiography and Definition of Valvular Heart Disease Stages

Procedures for echocardiography in ARIC at Visit 5, including reproducibility metrics, have been previously described, and were similar at Visit 7.(7) At both Visits 5 and 7, studies were acquired by certified sonographers using uniform imaging machines (Philips iE33, Koninklijke Philips, The Netherlands) and probes (Philips XMatrix) and acquisition protocols. Quantitative measures for studies from both visits were performed at the same dedicated Echocardiography Reading Center by trained analysts who were blinded to clinical information and in accordance with American Society of Echocardiography (ASE) recommendations.(8-10) At both Visits, all quantitative measures were over-read by study investigators who were staff cardiologists at the Brigham and Women's Hospital with COCATS level 3 advanced training in echocardiography and/or ASE Board Certification in Comprehensive Adult Echocardiography.

VHD stages were defined uniformly at both Visits 5 and 7 based on the ACC/AHA guideline recommendations and operationalized in this study as shown in **Table 1** (see Data Supplement for additional details).

Concordant with ACC/AHA VHD guidelines, mitral regurgitation (MR) was quantified based on the MR jet area (MRJA) to left atrial area (LAA) ratio. The MR color Doppler signal was traced on the systolic frame demonstrating the greatest jet extent, and LAA was measured at end-systole, in both the 4- and 2-chamber views. The larger MRJA:LAA ratio of the 4- and 2-chamber view values was used (7-9), and MR severity grade was increased by one grade if a wall-hugging eccentric jet was present. For aortic stenosis (AS), aortic valve (AV) peak jet velocity (Vmax) and velocity-time integral (VTI) and the LV outflow tract (LVOT) VTI by continuous-wave (CW) and pulsed wave (PW) Doppler respectively were acquired from the apical 5 chamber view, and LVOT diameter was measured in the parasternal long axis view. Aortic valve area (AVA) was calculated using the continuity equation as follows: AVA = $[CSA_{LVOT} * VTI_{LVOT}] / VTI_{AV}$, where CSA indicates LVOT cross-sectional area.(7, 10) Over-reading cardiologists performed qualitative assessments of aortic regurgitation (AR) severity based on AV color Doppler signal in the parasternal long- and short-axis views and in the apical 5- and 3-chamber view, and of mitral stenosis (MS) severity. Additionally, mitral valve area (MVA) was calculated from the pressure half-time derived from the mitral inflow deceleration time, with a MVA ≤ 1.5 cm^2 indicating moderate or greater MS.(11) Over-reading cardiologists also determined AV morphology (tricuspid versus bicuspid), the presence of mitral valve prolapse, and the presence of mitral annular calcification.

Among participants who attended Visit 5 but not Visit 7, those with a post-Visit 5 hospitalization or death certificate with a VHD ICD code (see Data Supplement for specific

codes) were classified as VHD Stage C/D at Visit 7. Those with an ICD procedure code for a VHD intervention post-Visit 5 were categorized as having a valve replacement or repair at Visit 7.

Prevalent and Incident Cardiovascular Events

ARIC cohort participants undergo surveillance for cardiovascular events through annual questionnaires and review of hospitalization discharge codes as previously described.(6) Coronary heart disease (CHD) events (definite or probable myocardial infarction (MI), fatal CHD, or coronary revascularization) were ascertained based on medical record abstraction and committee adjudication of hospitalizations with CHD related ICD-9 and ICD-10 codes.(6, 12) Prevalent CHD was defined as an adjudicated CHD event occurring before the Visit 5 date, while incident CHD was based on an adjudicated CHD event occurring after the Visit 5 date. Prevalent heart failure (HF) at Visit 5 was defined based on physician adjudicated HF hospitalization occurring since 2005 as previously published(13), ICD-9-CM 428 code for hospitalizations prior to 2005,(14) or HF self-report at Visits or on annual follow-up phone calls. Incident HF post-Visit 5 was ascertained based on ARIC adjudication of hospitalizations and death with HF-related ICD codes obtained by ARIC surveillance of hospital discharge as previously described.(13) Incident atrial fibrillation (AF) was ascertained through hospitalizations with ICD-9 427.31 or ICD-10 I48.91 discharge codes through the end of 2017.(15, 16) A potential stroke hospitalization was considered for validation if the discharge diagnosis contained a cerebrovascular disease diagnosis code (ICD-9 codes 430 – 438), if the discharge summary included a keyword related to cerebrovascular procedure or disease, or if there was imaging evidence of cerebrovascular disease. Validation procedures followed the

National Survey of Stroke criteria for stroke definition as previously described. (17, 18) Death was ascertained using the National Death Index. The end date for follow-up was December 31, 2019, except for 697 participants from Jackson center whose follow-up was through December 31, 2017 due to administrative reasons.

Clinical Covariates and Biomarkers

Hypertension was ascertained based on participant report of blood pressure medication use or blood pressure ≥140/90 mm Hg at any ARIC visit. Diabetes mellitus was ascertained based on self-report of a physician diagnosis of diabetes mellitus, antidiabetic medication use, fasting glucose ≥126 mg/dL, or non-fasting glucose ≥200 mg/dL at any ARIC visit. Body mass index was calculated from weight and height assessed at Visit 5. Frailty was assessed at Visit 5 using Fried criteria, which incorporates gait speed, grip strength, low energy expenditure, weight loss, and exhaustion.(19) Estimated glomerular filtration rate estimated (eGFR) was calculated using the CKD Epidemiology Collaboration (CKD-EPI) equation. (20) NT-proBNP was measured using electrochemiluminescent immunoassay (Roche Diagnostics), with a lower detection limit of ≤5 ng/mL. (21) Hs-TnT was measured using a highly sensitive assay (Elecsys Troponin T, Roche Diagnostics), and the limit of the blank was 3 ng/L.(21, 22)

Statistical Methods

Participants were classified based on the most severe valve stage of the following valve lesions: AS, AR, MS, MR. VHD stage prevalence was also described stratified by age category (65 - 70, 71 - 75, 76 - 80, >80 years old), sex, and race. Multivariable linear regression models were used to relate VHD stages to concentrations of NT-proBNP and hs-TnT at Visit 5. Values

of both biomarkers were log-transformed to achieve normality. Multivariable Cox proportional hazard models were used to assess the relationship of the VHD stage at Visit 5 with incident CHD, HF, AF, stroke, or death. Initial models adjusted for age, sex, and race. Subsequent models further adjusted for hypertension, diabetes, prior MI, HF, body mass index, Field Center, and systolic blood pressure at Visit 5. A sensitivity analysis was done by adding eGFR and low-density lipoprotein (LDL) to model 2. For each endpoint, models excluded participants with the prevalent condition at Visit 5, and participants with prior valve replacement. Analyses were performed by VHD stage overall and for stage of each valvular lesion individually (AS, AR, MS, and MS). To assess the potential impact of non-random Visit 5 non-attendance on the survival analyses, we performed a sensitivity analysis incorporating inverse probability of attrition weights (IPAW).(23, 24) Visit 5 non-attendance was modeled among ARIC participants alive at the initiation of Visit 5 using the following covariates from Visit 1: age, gender, race, study center, systolic and diastolic blood pressure, heart rate, smoking and drinking status, diabetes, hypertension, and eGFR . The resulting calculated weights were incorporated into the multivariable Cox proportional hazards models.

We then assessed transitions in VHD stage between Visit 5 and Visit 7 overall, and by the valvular lesion. We employed IPAW to account for Visit 7 non-attendance among ARIC participants who attended Visit 5 and were alive at Visit 7. Visit 7 non-attendance was modeled among participants alive through the end of Visit 7 using the following covariates from Visit 5: age, gender, race, study center, frailty, cancer, diabetes, hypertension, and prevalent HF. Distinct covariates were selected for calculating IPAW at Visit 5 compared to Visit 1 as the causes of non-attendance may be different at different ages. The resulting calculated weights were

incorporated to estimate VHD stage prevalence at Visit 7 among all Visit 5 participants alive at the time of Visit 7.

All analyses were performed using STATA 16. Two-sided P-values of less than 0.05 were considered significant.

Results

Prevalence and Correlates of VHD Stages at Study Visit 5

The mean age of the study sample at Visit 5 was 76±5 years, 42% were male, and 22% were black **(Table 2).** Stage A VHD was present in 39%, Stage B in 17%, and Stage C/D in 1.1%, while 0.7% had previously undergone valve replacement or repair (**Figure 2A**). VHD was absent (i.e., Stage 0) in 43 % of participants. Older age was associated with a higher prevalence of all VHD stages **(Figure 2B**). The prevalence of VHD was 44% and 53% in Black men and women respectively, and was 56% and 61% in White men and women respectively (**Figure 2C**). Greater VHD stage was characterized by a higher prevalence of cardiovascular risk factors, such as hypertension, diabetes, obesity, and chronic kidney disease, and of prevalent CVD, including prior MI, stroke, and AF **(Table 2).** In analyses adjusted for age, sex, race, history of hypertension, diabetes, MI, HF, body mass index, Field Center, and systolic blood pressure at Visit 5, greater VHD stage was associated with higher concentrations of NT-proBNP and hs-TnT **(Figure 3)**. Concentrations of these biomarkers were higher in both Stage A and in Stage B compared to participants free of VHD. Similar associations were observed within each valvular lesion **(Supplemental Figure 1)**.

Stages of Aortic Valve Disease

Stage A AS was present in 15% (bicuspid valve in 11 participants), Stage B in 4.1% (3.8% mild AS, 0.3% moderate AS), Stage C (asymptomatic severe AS) in 27 participants (0.4%), and Stage D (symptomatic severe AS) in 26 participants (0.4%), while 32 participants had undergone prior AV replacement (**Figure 2D**). Stage C/D AS was responsible for 80% of participants with Stage C/D VHD overall. Mild aortic regurgitation (Stage B1) was present in 10.2% of participants, and moderate aortic regurgitation was identified in 0.5% (Stage B2). No participants had severe (Stage C) aortic regurgitation. Stage B aortic regurgitation was responsible for 62% of participants with Stage B VHD overall.

Stages of Mitral Valve Disease

Stage A MR was present in 39% (mild MR in 1,740 [28.5%]), Stage B MR (moderate MR in 4%), and Stage C/D MR (severe MR) in 12 participants (0.2%; 1 with symptoms [Stage D], 4 with asymptomatic reduced LVEF), while 13 participants had a prior MV repair or replacement (**Figure 2D**). Stage A MR was responsible, either alone or in combination with other Stage A lesions, for 80% of the participants who had overall VHD Stage A. Mitral stenosis was rare in this sample, with rheumatic deformity identified in only 1 participant, and qualitative mild stenosis related to calcification noted in 11 participants (0.2% prevalence; only 1 with calculated MVA $\leq$ 1.5 cm^2).

VHD Stages and Incident Cardiovascular Events

Over a median follow-up of 6.5 (IQR, 3.7-7.7) years, 1,295 participants died, incident HF occurred in 553 of those free of HF at Visit 5, incident CHD in 300 of those free of CHD at Visit 5, and incident stroke in 250 of those free of stroke at Visit 5. Over a median follow-up of 5.5 (IQR, 4.8-5.9) years, incident AF occurred in 564 of those free of AF at Visit 5. Among those

who died, 25 had death certificates with VHD related ICD diagnostic codes. In models adjusting for demographics and cardiovascular co-morbidities, a graded association was observed between Stage A, B, and C/D VHD and risk of all-cause mortality, incident HF, incident AF, and incident CHD, but not with risk of incident stroke (**Figure 4**, **Table 3**). Notably, compared to those free of VHD (VHD Stage 0), each stage was associated with a heightened risk of these outcomes in adjusted models, including Stage A **(Supplemental Figure 2).** Similar associations were observed after incorporating IPAW to account for Visit 5 non-attendance **(Supplemental Table 1)** and after adding eGFR and LDL to the model 2 **(Supplemental Figure 3).** Similar associations were also observed for stages of each valvular lesion **(Supplemental Table 2, Supplemental Figure 4**), and after excluding participants who had another concomitant valvular lesion with a higher stage **(Supplemental Figure 5)**

Among 1,010 participants with Stage B VHD, at least two valvular lesions were present in 60% (the additional lesion being Stage A in 49% and Stage B in 11%). Compared to Stage B participants with only 1 Stage B lesion, involvement of a second valvular lesion was associated with a trend toward higher risk for the composite of death, HF, CHD, AF, or stroke (HR 1.34 [95% CI 1.00- 1.80], p= 0.049 in the model adjusted for demographics; HR 1.38[1.00- 1.89], p= 0.048 in the fully adjusted model; **Supplemental Figure 6**).

Progression of VHD Stage from Visit 5 to Visit 7

During the 6.6 (IQR, 6.1-7.0) years between Visit 5 and Visit 7, 1,223 participants died, and 2,896 (60%) of surviving participants chose to attend and underwent repeat echocardiography. Among those without Visit 7 echocardiography, 25 participants died with VHD-related death certificate ICD codes, 146 were hospitalized with VHD-related ICD codes,

and 21 were hospitalized with VHD procedure-related ICD codes (**Figure 1**). Among the 2896 with repeat echocardiography at Visit 7, mean age was 81± 4 years, 57% were women, and 23% were Black. Stage A VHD was present in 20%, Stage B in 10%, and Stage C/D in 4% (**Figure 5; Supplemental Figure 7**). After incorporating IPAW to account for Visit 7 non-attendance, the prevalence of VHD Stage 0 decreased between Visits 5 and 7 (43 to 24% respectively), as did the prevalence of Stage A VHD (39 to 31% respectively), while the prevalence of Stage C/D VHD increased (1 to 7% respectively) as did the prevalence of valve replacement or repair (1 to 2% respectively). No major changes were observed in the prevalence of Stage B VHD (17 to 16% respectively; **Figure 5a**). Similar reductions in the proportion of participants without VHD stage from Visit 5 to Visit 7 were observed for each valve lesion, and similar increases in the prevalence of Stage C/D VHD stage were observed for AS and MR (**Supplemental Figure 8**).

The magnitude of decline in prevalence of VHD Stage 0 and increase in Stage C/D prevalence was greater at older age, despite higher mortality between Visits 5 and 7 among older participants (**Figure 5b**). Among participants >80 years of age at Visit 5 the prevalence of VHD Stage C/D increased from 2 to 12% over 6 years, compared to an increase from 0.7 to 4% among those <70 years of age at Visit 5 and despite a much higher mortality between Visits 5 and 7 among the older group (39 vs. 9%). After accounting for age, changes in prevalence of VHD Stages 0 and C/D were similar among subgroups defined by gender and race (**Figure 5c**).

Discussion

This analysis is one of the first to quantify the prevalence, prognostic relevance, and progression of ACC/AHA VHD stages in a large, diverse, community-based cohort of persons in late-life. We report three major novel findings. First, less than half of older adults are free of VHD stage, with 39% having Stage A and 17% having Stage B VHD. Second, compared to

those free of VHD, a higher VHD stage was associated with a graded increase in risk of incident HF, CHD, AF, and mortality after adjustment for traditional cardiovascular risk factors. Even Stage A VHD was associated with heightened risk for adverse cardiovascular events compared to those free of VHD. Third, VHD stages are progressive, and accelerate in late-life, with a decline in the proportion free of VHD stage from 43 to 24% and an increase in the prevalence of Stage C/D VHD from 1 to 7% over 6 years. These findings, which capture the range of sub-severe VHD in the community, highlight the scope and pace of progression of VHD among older adults.

Numerous prior studies have documented the prevalence of individual valvular lesions,(25-32) frequently using clinically referred samples and focusing on greater degrees of lesion severity.(33-36) In contrast, Nkomo et al.'s landmark large pooled analysis of several population-based studies identified moderate or severe VHD in 2.5%, with marked increase in prevalence with age such that 13 % of those ≥75 years old had moderate or severe VHD.(1) More recently, the OxVALVE study of 2,500 patients ≥65 years old recruited from primary care clinics demonstrated a prevalence of mild VHD in 44% and moderate or severe VHD in 6.4%.(2)

In contrast to prior studies, which largely characterize VHD lesions as mild or significant (moderate or severe), our study is the first to our knowledge to implement the ACC/AHA VHD stages framework. While the construct of disease stages has been incorporated into HF guidelines for two decades,(37) its incorporation into VHD guidelines is relatively recent.(4) In addition to emphasizing the progressive nature of valvular lesions, articulation of VHD stages also provides a framework for quantifying the population burden of lesser degrees of valvular dysfunction. Our study now extends upon prior studies by evaluating VHD stages in a diverse, community-based sample of older-adults, defining the association of non-severe VHD with CV

outcomes beyond mortality, and quantifying the progression in VHD stages over 6 years in late-life.

In an elderly community-based cohort, our study found that only 43% were free of VHD, similar to that observed in the OxVALVE study (49%), which included aortic sclerosis and mild mitral regurgitation.(2) The most common VHD stages in our study were Stage A (at risk) based on valvular deformity, calcification, aortic sclerosis, or mild MR, followed by Stage B (progressive VHD), which included other mild and moderate valvular lesions. The prevalence of the Stage C or D VHD (symptomatic and asymptomatic severe valvular lesions) was present in 1% of participants at Visit 5 in our study, lower than observed in OxVALVE (6.4%) and the Nkomo et al. study (2.5%). (1, 2) Importantly, the prevalence in our study increased to 7% at Visit 7, more consistent with OxValve and Nkomo et al. The lower prevalence at Visit 5 may therefore be related to healthy attendance bias, leading to underestimation of the true prevalence. Furthermore, the OxVALVE study sampled patients from UK primary care clinics which may have resulted in over-sampling of patients with VHD compared to a community-based cohort. Between-study differences in VHD definition likely also contribute to differences in prevalence estimates. For example, significant AS in the Nkomo et al. study was based on an AVA $\leq$1.5 cm^2 assessed variably by each component cohort.(1, 2, 38)

Severe AS and MR are the most common severe valvular lesions in western countries (39) and are associated with risk of all-cause mortality even when asymptomatic.(30, 40, 41) Consistent with these data, Stage C/D VHD in our study was mainly due to AS and MR and was associated with a markedly increased risk of mortality and incident CVD compared to those free of VHD. Importantly, Stage A and Stage B VHD were also associated with higher concentrations of biomarkers of myocardial stress (NT-prBNP) and injury (hsTn-T), and with a heightened risk

mortality, MI, AF, and HF in a graded fashion even after adjustment for common cardiovascular risk factors. These findings are consistent with prior studies demonstrating the association of mild and moderate AS, aortic sclerosis in the absence of stenosis, mild and moderate MR, and mild degenerative mitral stenosis with a higher risk of mortality and CV events such as MI.(42-47) It remains unclear whether Stage A, or even Stage B, VHD is etiologically related to mortality or incident CVD, or is instead a marker of unmeasured cardiovascular risk factors.(46) Indeed, modest alterations in valve function, such as aortic sclerosis and mild stenosis, are associated with a worse cardiovascular health score after adjusting for demographics.(48)

Limited data exist regarding the progression of VHD, and of VHD stages, particularly in late life when both prevalence and incidence of VHD are greatest. Previous studies have found that progression from aortic sclerosis to clinically significant stenosis occurs in 5.4% of patients over seven years,(49, 50) while progression from mild to moderate AS to severe AS occurs in upto 47% of asymptomatic patients over five years. (47) Progression in MR severity over time appears more variable, likely because of the diverse etiologies of MR and numerous factors influencing regurgitation severity.(51) We observed a marked four to seven fold increase in the prevalence of Stage C/D VHD over six years in late-life, from a mean age of 76±5 to 81±4 years. These increases were driven by increases in Stage C/D prevalence of AS and MR, which demonstrated five- and ten-fold increase, respectively. Importantly, the rate of progression in VHD was greater at older ages, despite the greater competing risk of death. This accelerated progression highlights the importance of surveillance for VHD and of developing approaches targeting prevention and/or mitigation of VHD in late life.

This study has several limitations. Non-attendance of surviving ARIC participants at Visit 5 may introduce healthy selection bias and limit generalizability. A sensitivity analysis

incorporating IPAW demonstrated similar results. Non-attendance at Visit 7 (30%) was non-random and may therefore introduce attrition bias and underestimation of the prevalence of Stage C/D VHD at Visit 7. We therefore performed analyses incorporating IPAW to account for Visit 7 non-attendance. Furthermore, among Visit 7 non-attendees, we assessed death and hospitalization ICD codes between Visits 5 and 7 to detect clinical progression. The operational definition of AR and MS was based primarily on qualitative assessment by COCATS level III trained cardiologists using ASE criteria. PISA-based measures of MR severity were not available for the classification of progressive and severe MR. Information regarding the type and cause of MR was also not available. The low prevalence of MS limited our ability to assess the association of MS stage with clinical outcomes. Details of the primary indication and the type of AV and MV interventions were not available. Finally, residual confounding can't be excluded due to the observational nature of the design.

Conclusions

In a diverse community-based cohort of older adults, subclinical VHD is common, with 39% at risk of VHD (Stage A) and 17% with progressive VHD (Stage B). Stage A and Stage B VHD are associated with a heightened risk of incident cardiovascular events independent of traditional cardiovascular risk factors. VHD stages progress over six years in late life, with a several-fold increase in the prevalence of severe VHD (Stage C/D). These findings clarify the burden of VHD in late-life and highlight the public health importance of interventions to mitigate VHD progression.

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Table 1: Operationalization of VHD stages in ARIC

	Aortic stenosis	Aortic regurgitation	Mitral stenosis	Mitral regurgitation
Stage A: ***At risk***	**Sclerosis:** V_{max} from 1.5 to <2.0m/sec **or** Bicuspid valve	**Sclerosis**: V_{max} from 1.5 to <2.0m/sec **or** Bicuspid valve	Mitral annular calcification **or** **Rheumatic features:** rare	Mitral annular calcification **or** **Mitral valve prolapse (MVP)** **or** **Mild MR:** (^MRJA:LAA ratio 5 - 20%)
Stage B: ***Progressive***	**Mild AS:** V_{max} from 2.0 to <3 m/sec **or** **Moderate AS:** V_{max} from 3 to <4 m/sec or mean ΔP from 30 to< 40 mmHg	**Mild AR:** (Qualitative) **or** **Moderate AR:** **(**Qualitative)	**Qualitative MS** and $^{\$}$Calculated MVA >1.5 cm^2	**Moderate MR:** (^MRJA:LAA ratio 20-40%) or eccentric jet with mild M.R.)
Stage C: ***Asymptomatic Severe***	**Severe AS:**(V_{max} ≥4.0 m/sec or mean ΔP ≥ 40 mmHg) + **Stage C1:** LVEF ≥50% or **Stage C2:** LVEF <50% **or** Asymptomatic low flow*	**Severe** AR (Qualitative) + **Stage C1:** LVEF ≥55%, and LVESD ≤5 cm or **Stage C2:** LVEF <55% or LVESD > 5 cm	**Qualitative MS** and $^{\$}$Calculated MVA ≤ 1.5 cm^2	**Severe MR:** (^MRJA:LAA ratio ≥40% or eccentric jet with moderate M.R.) + **Stage C1:** LVEF>60% and LVESD <4.0cm or **Stage C2:** LVEF ≤60% or LVESD≥4.0cm
Stage D: ***Symptomatic Severe***	Symptoms* or Angina + **Stage D1: Severe AS** ± LVEF ≥50% or **Stage D2: low flow** and LVEF <50%, or **Stage D3: low flow** and LVEF ≥50%	Symptoms* + **Severe AR** (Qualitative)	Symptoms* + $^{\$}$Calculated MVA ≤ 1.5 cm^2	Symptoms* + **Severe MR**

V_{max}: Peak aortic valve velocity by Doppler echocardiography, Mean ΔP: The mean pressure gradient across the aortic valve by Doppler. *Low flow: V_{max} < 4.0 m/sec and AVA ≤1.0cm^2. *Symptoms: dyspnea, exhaustion, or heart failure; $Calculated Mitral valve area (MVA) = 220 / Pressure half-time (PHT) where Pressure half-time (PHT) = mitral inflow deceleration time x 0.29; ^MRJA:LAA ratio: the ratio between mitral regurgitation jet area and left atrial area

Table 2: Baseline characteristics at Visit 5 by VHD stage

	Overall	Stage 0	Stage A	Stage B	Stage C/D	
N	6118	n=2640	n=2362	n=1010	n=66	P-value
Age, years	76 ± 5	75 ± 5	76 ± 5	77 ± 5	78 ± 5	<0.001
Male, n (%)	2576 (42%)	1168 (44%)	910 (39%)	440 (44%)	32 (48%)	<0.001
Black, n (%)	1334 (22%)	684 (26 %)	483 (20%)	148 (15%)	11 (17%)	<0.001
Center, n (%)						
Forsyth County, NC	1416 (23%)	541 (20%)	586 (25%)	263 (26%)	21 (32%)	<0.001
Jackson, MS	1214 (20%)	624 (24%)	439 (19%)	134 (13%)	9 (14%)	
Minneapolis, MN	1823 (30%)	851 (32%)	628 (27%)	316 (31%)	13 (20 %)	
Washington County, MD	1665 (27%)	624 (24%)	709 (30%)	297 (29%)	23 (35%)	
Ever Smoker, n (%)	3764 (62%)	1666 (63%)	1388(59%)	648 (64%)	38 (58%)	0.003
Current Smoker, n (%)	349 (6%)	158 (6 %)	147 (6 %)	40 (4 %)	3 (5 %)	0.07
HTN, n (%)	5103 (83%)	2142 (81%)	2003 (85%)	864 (86 %)	58 (88%)	<0.001
DM, n (%)	2301 (38%)	1048 (40%)	884 (37%)	322 (32%)	32 (48%)	<0.001
CKD, n (%)	1705 (28%)	659 (25%)	677 (29%)	329 (33%)	24 (37%)	<0.001
CHD, n (%)	939 (16%)	314 (12%)	395 (17%)	193 (19%)	18 (28%)	<0.001
MI, n (%)	709 (12%)	261 (11%)	285 (13%)	138 (14%)	14 (23%)	<0.001
HF, n (%)	955 (16%)	328 (12%)	389 (16%)	192 (19%)	18 (27%)	<0.001
Stroke, n (%)	224 (4%)	69 (3 %)	98 (4%)	49 (5 %)	3 (5 %)	0.003
AF, n (%)	442 (7%)	120 (5%)	191 (8 %)	103 (10 %)	10 (15 %)	<0.001
BMI, kg/m2	29 ± 6	29 ± 6	29 ± 6	28 ± 5	28 ± 5	<0.001
SBP, mmHg	130 ± 18	129 ± 17	131 ± 18	132 ± 19	132 ± 20	<0.001
DBP, mmHg	67 ± 11	67 ± 10	66 ± 11	65 ± 11	65 ± 15	<0.001
Pulse Pressure, mmHg	64 ± 15	61 ± 13	65 ± 15	67 ± 16	67 ± 15	<0.001
Heart rate, bpm	65 ± 11	66 ± 11	65 ± 11	64 ± 11	64 ± 11	<0.001
Hemoglobin A1c (%)	5.9 ± 0.8	6.0 ± 0.9	5.9 ± 0.8	5.8 ± 0.7	6.0 ± 0.9	<0.001
eGFR,mL·min-1^{-1}.$73m^{-2}$	70 ± 17	71 ± 17	70 ± 17	67 ± 18	65 ± 18	<0.001
LDL (mg/dL)	104 ± 35	106 ± 35	103 ± 33	103 ± 35	99 ± 34	0.007
HDL (mg/dL)	52 ± 14	52 ± 14	53 ± 14	52 ± 13	51 ± 15	0.88

Hypertension (HTN), diabetes mellites (DM), chronic kidney disease (CKD), coronary heart disease (CHD), myocardial infarction (MI), heart failure (HF), atrial fibrillation (AF), body mass index (BMI), systolic blood pressure (SBP), Diastolic blood pressure (DBP), estimated glomerular filtration rate (eGFR), low density lipoprotein (LDL), high density lipoprotein (HDL)

Table 3: Association of VHD stages at ARIC Visit 5 with incident death or cardiovascular event.

		N	Events	Rate per 100 PY	HR (Model 1)	P-value	HR (Model 2)	P-value
Death	Stage 0	2,640	420(16%)	2.4 (2.2-2.6)	Reference group			
	Stage A	2,362	515(22%)	3.3 (3.1-3.6)	1.3(1.1-1.4)	**<0.001**	1.3(1.1-1.4)	**0.001**
	Stage B	1,010	306(30%)	4.8(4.3-5.3)	1.5(1.3-1.7)	**<0.001**	1.4(1.2-1.7)	**<0.001**
	Stage C/D	66	38 (58%)	9.6(7.013.1)	3.0(2.2-4.2)	**<0.001**	2.4(1.7-3.5)	**<0.001**
HF	Stage 0	2,545	153(6%)	0.9(0.8-1.1)	Reference group			
	Stage A	2,219	248(11%)	1.7(1.5-2.0)	1.8(1.5-2.2)	**<0.001**	1.9(1.5-2.4)	**<0.001**
	Stage B	909	136(15%)	2.4(2.0-2.9)	2.2(1.7-2.8)	**<0.001**	2.3(1.8-3.0)	**<0.001**
	Stage C/D	56	16(29%)	5.4(3.3-8.9)	5.0(3.0-8.4)	**<0.001**	5.1(3.0-8.6)	**<0.001**
AF	Stage 0	2,495	186(7%)	1.4(1.2-1.6)	Reference group			
	Stage A	2,115	238(11%)	2.2(1.9-2.5)	1.5(1.2-1.8)	**<0.001**	1.4(1.1-1.7)	**0.001**
	Stage B	864	128(15%)	3.0(2.5-3.6)	1.7(1.4-2.2)	**<0.001**	1.7(1.3-2.1)	**<0.001**
	Stage C/D	53	12 (23%)	5.2(3.0-9.2)	3.1(1.7-5.6)	**<0.001**	2.7(1.5-5.1)	**0.001**
CHD	Stage 0	2,359	106(4%)	0.7(0.6-0.8)	Reference group			
	Stage A	2,019	123(6%)	0.9(0.8-1.1)	1.4(1.0-1.8)	**0.015**	1.4(1.0-1.8)	**0.027**
	Stage B	842	64(8%)	1.2(1.0-1.6)	1.6(1.2-2.2)	**0.003**	1.7(1.2-2.3)	**0.002**
	Stage C/D	50	7(14%)	2.6(1.2-5.4)	3.2(1.5-7)	**0.003**	3.1(1.4-6.8)	**0.004**
Stroke	Stage 0	2,579	102(4%)	0.6(0.5-0.7)	Reference group			
	Stage A	2,276	96(4%)	0.7(0.5-0.8)	1.0(0.8-1.4)	0.842	1.0(0.8-1.4)	0.757
	Stage B	967	50(5%)	0.8(0.6-1.1)	1.2(0.8-1.7)	0.382	1.2(0.8-1.7)	0.408
	Stage C/D	64	2(3%)	0.5(0.1-2.1)	0.7(0.2-3.0)	0.669	0.4(0.1-2.5)	0.300

Event rates are per 100 person-years. HRs are adjusted. Model 1: adjusted for age, sex, and race; Model 2: adjusted for age, sex, race, Field Center, hypertension, diabetes, prior myocardial infarction, heart failure, body mass index, and systolic blood pressure.

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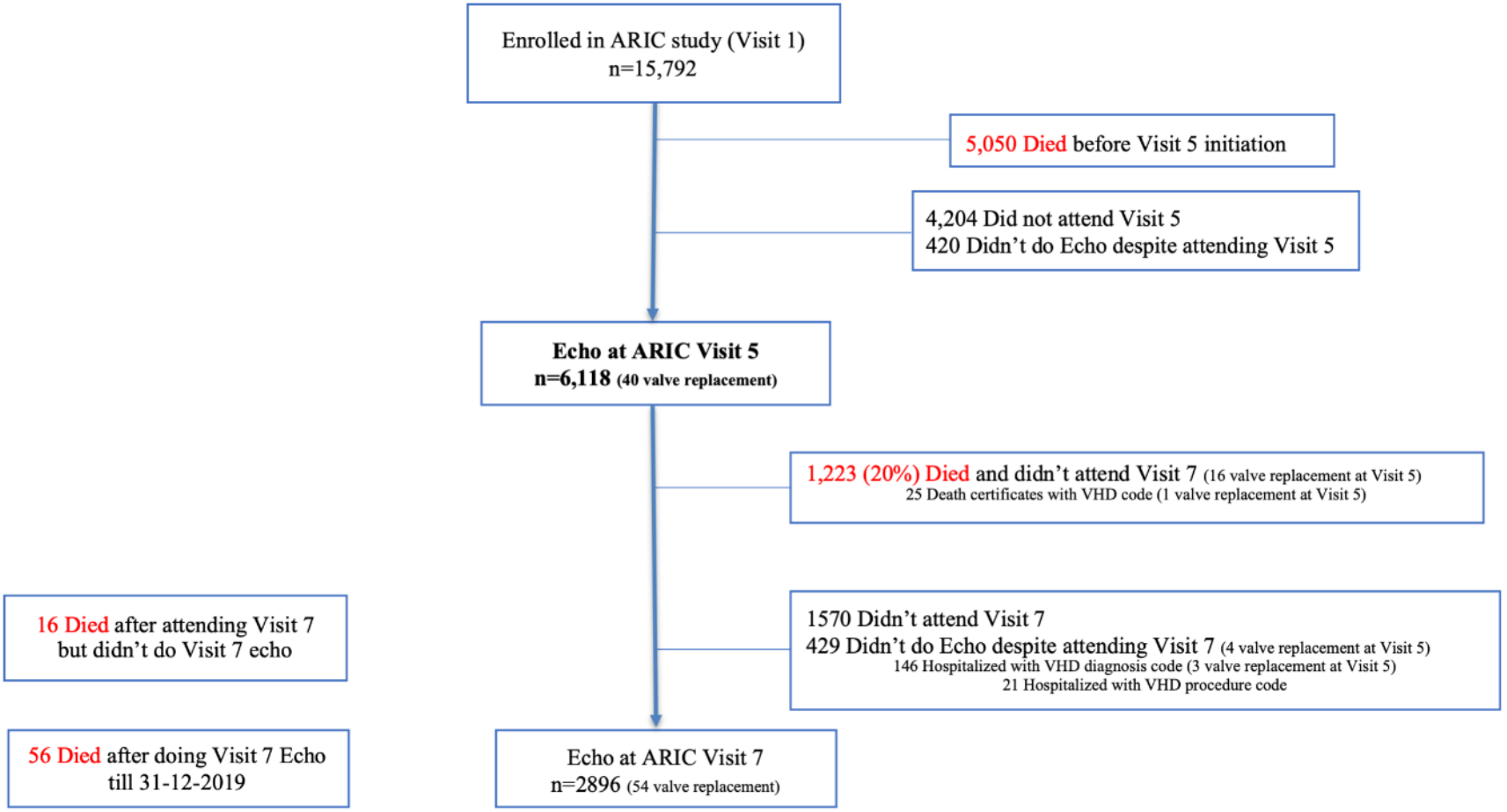
Figure 5. Transitions of VHD stages over 6.6 years from ARIC Visit 5 (mean age 76±5 years) to Visit 7 (mean age 81± 4 years) overall (Panel A), by age category (Panel B), and by race and gender groups (Panel C).

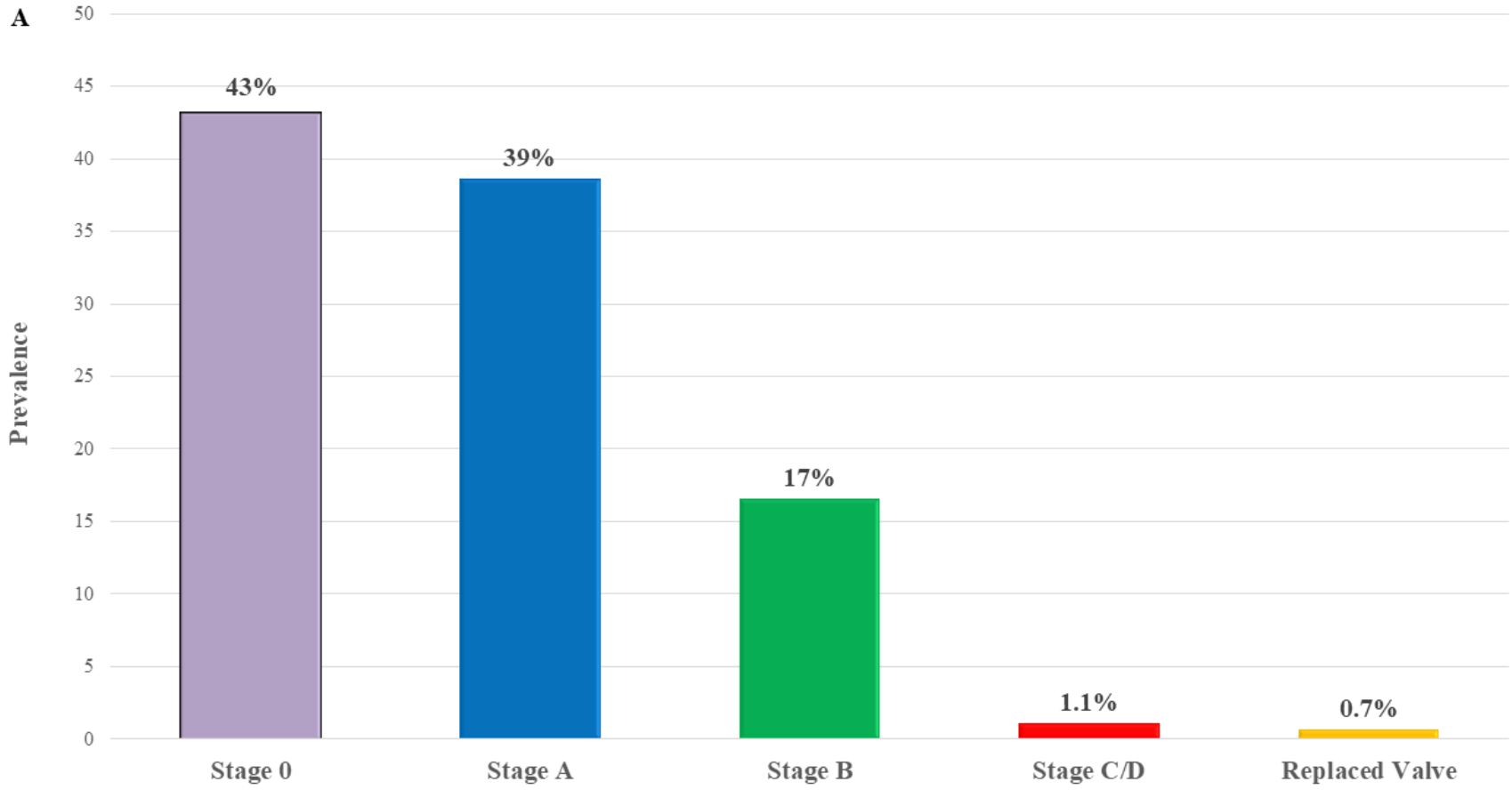
Sankey diagram in Panel A demonstrates the transition in VHD stage from Visit 5 to Visit 7 (left 2 columns). Right-most column demonstrates prevalence of VHD stages at Visit 7 using IPAW to account for Visit 7 non-attendance. For VHD transitions by age group (Panel B) and race and gender groups (Panel C), Visit 7 prevalence estimates are IPAW-adjusted. For VHD transitions by race and gender groups (Panel C), prevalence estimates are age adjusted.

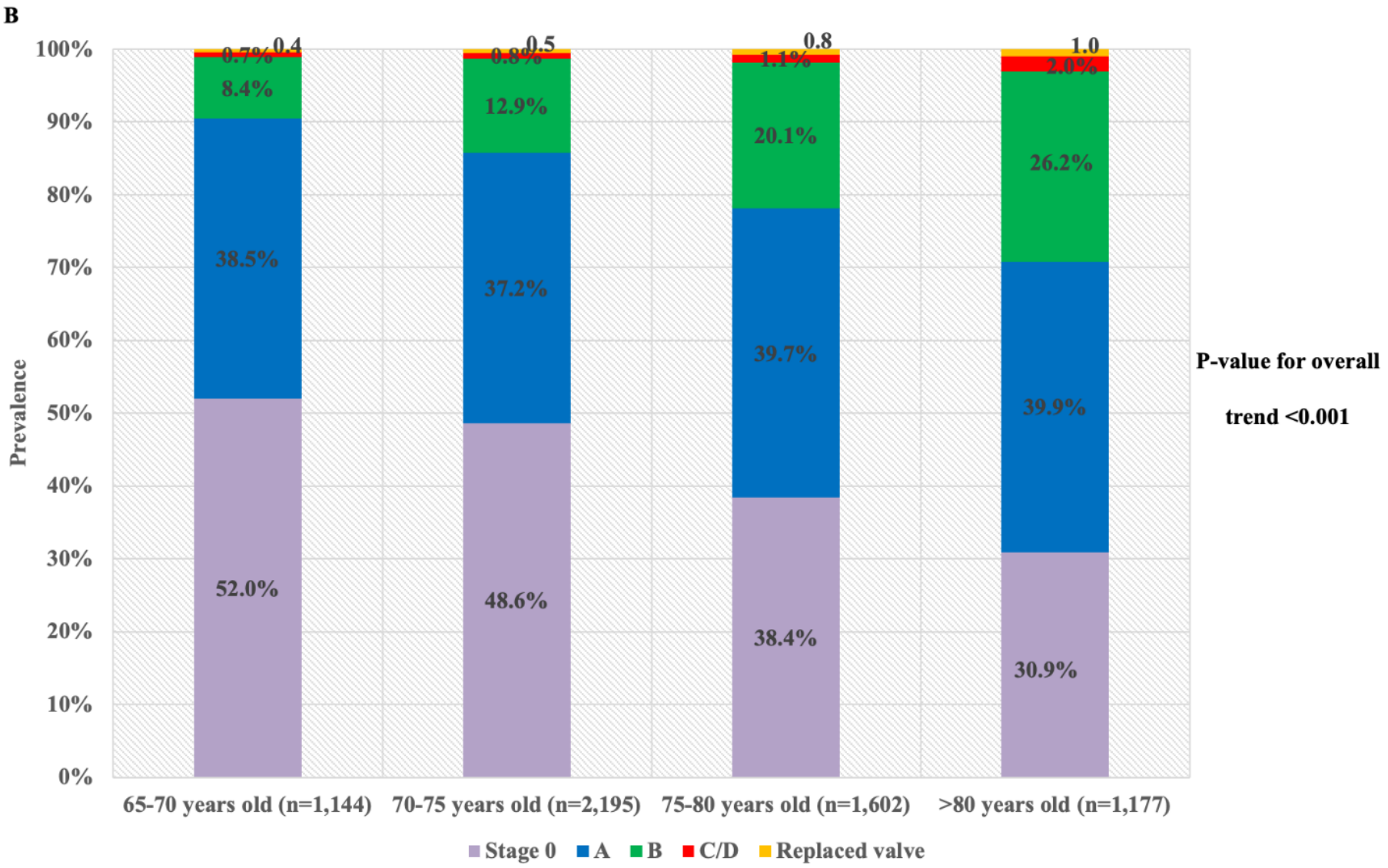
Figure 1: Flowchart of study participants

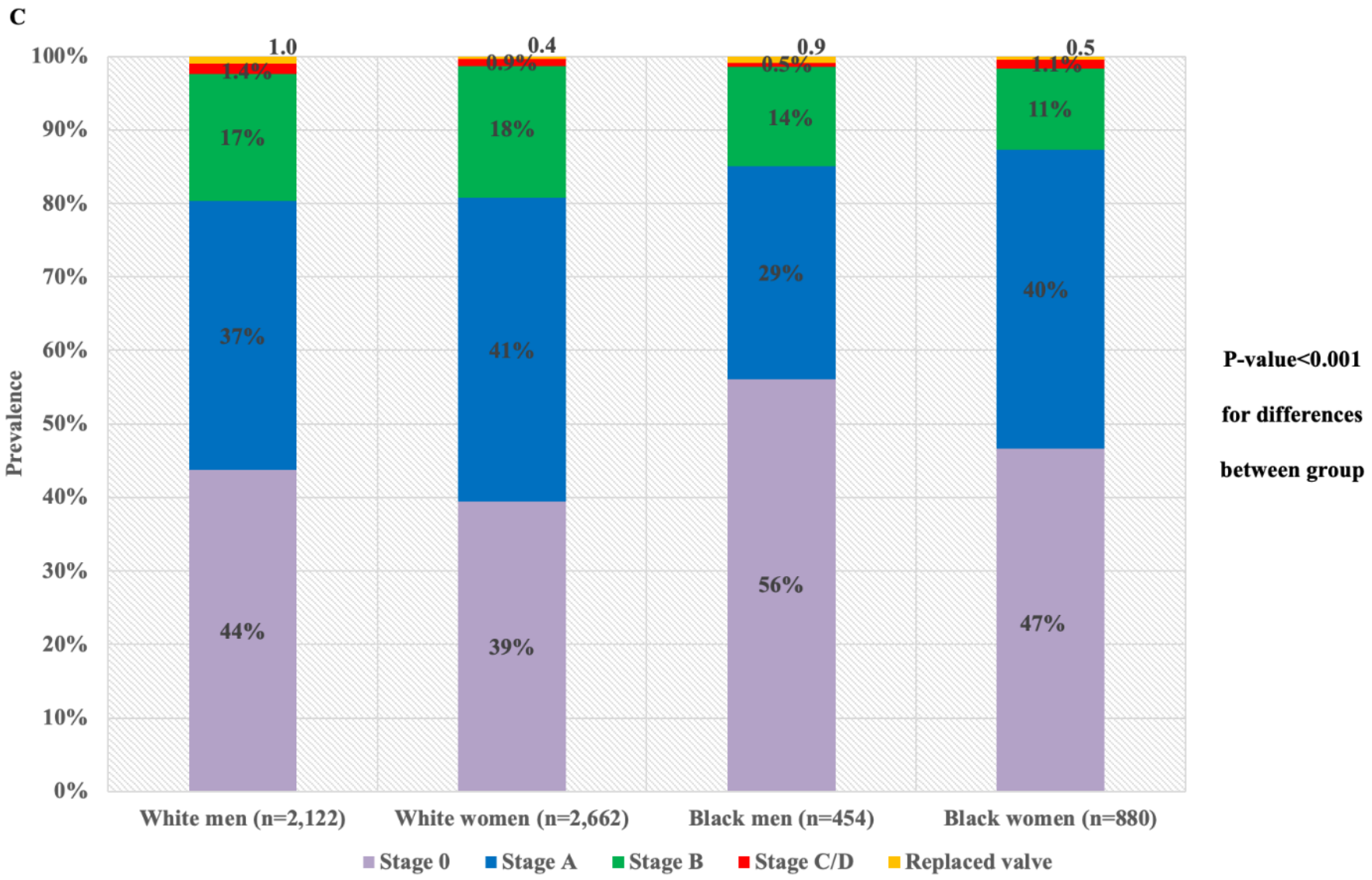


The bold text box represents study population, left side boxes showed the mortality in the corresponding right side boxes

Figure 2: Prevalence of VHD stages in ARIC at Visit 5 (n=6,118; mean age 76±5 years) overall (Panel A), by age category (Panel B), by race and gender groups (Panel C), and by valve lesion (Panel D). Prevalence by race and gender group is age-adjusted.




B
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Prevalence
0.7%
0.4
8.4%
38.5%
52.0%
0.8%
0.5
12.9%
37.2%
48.6%
1.1%
0.8
20.1%
39.7%
38.4%
2.0%
1.0
26.2%
39.9%
30.9%
P-value for overall
trend <0.001
65-70 years old (n=1,144)
70-75 years old (n=2,195)
75-80 years old (n=1,602)
>80 years old (n=1,177)
Stage 0
A
B
C/D
Replaced valve


C
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Prevalence
1.0
1.4%
17%
37%
44%
0.4
0.9%
18%
41%
39%
0.9
0.5%
14%
29%
56%
0.5
1.1%
11%
40%
47%
White men (n=2,122)
White women (n=2,662)
Black men (n=454)
Black women (n=880)
Stage 0
Stage A
Stage B
Stage C/D
Replaced valve
P-value<0.001
for differences
between group

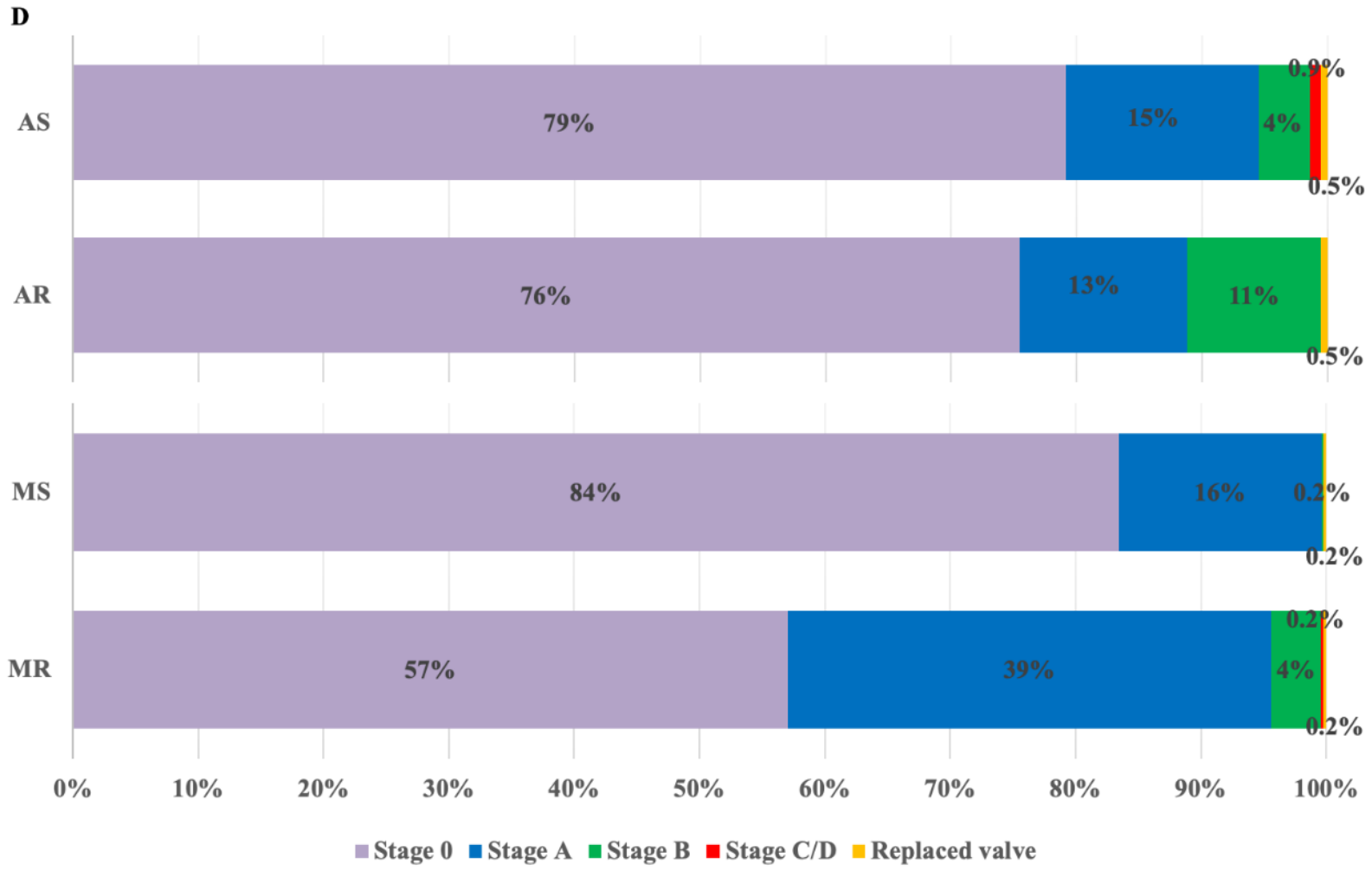
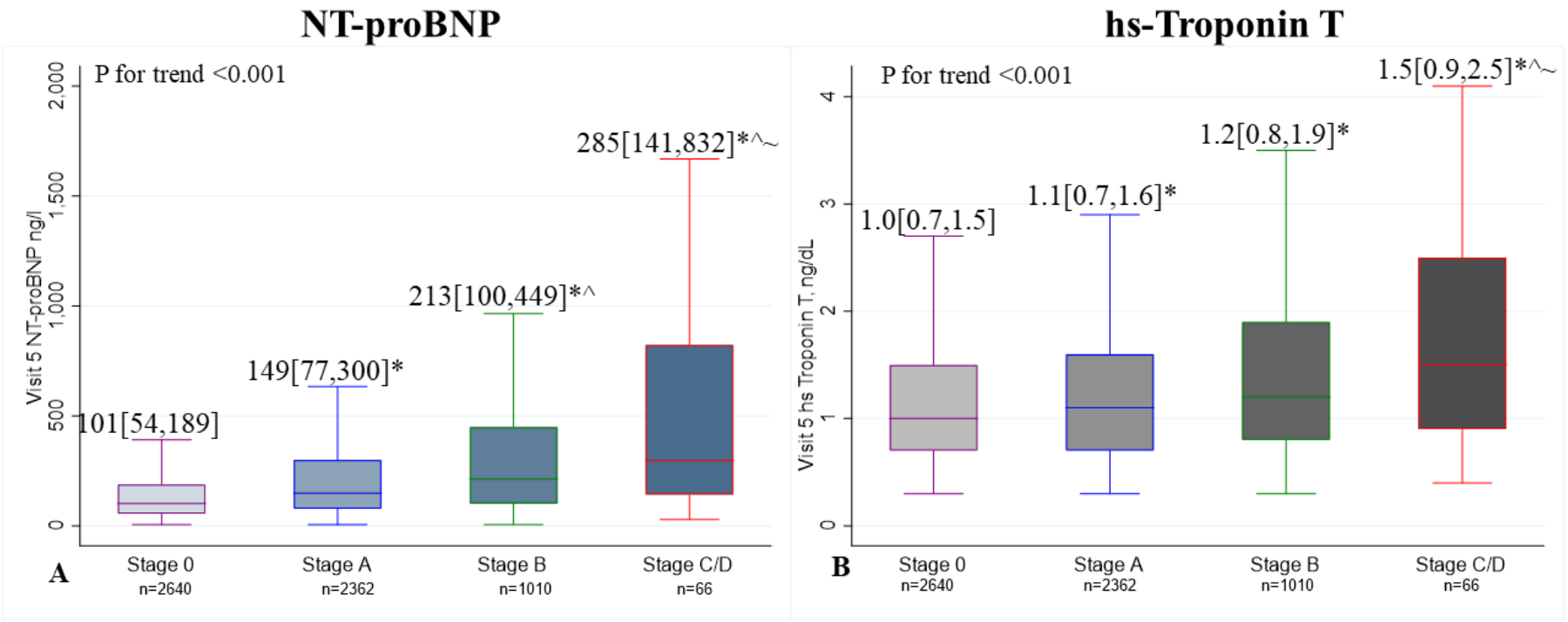
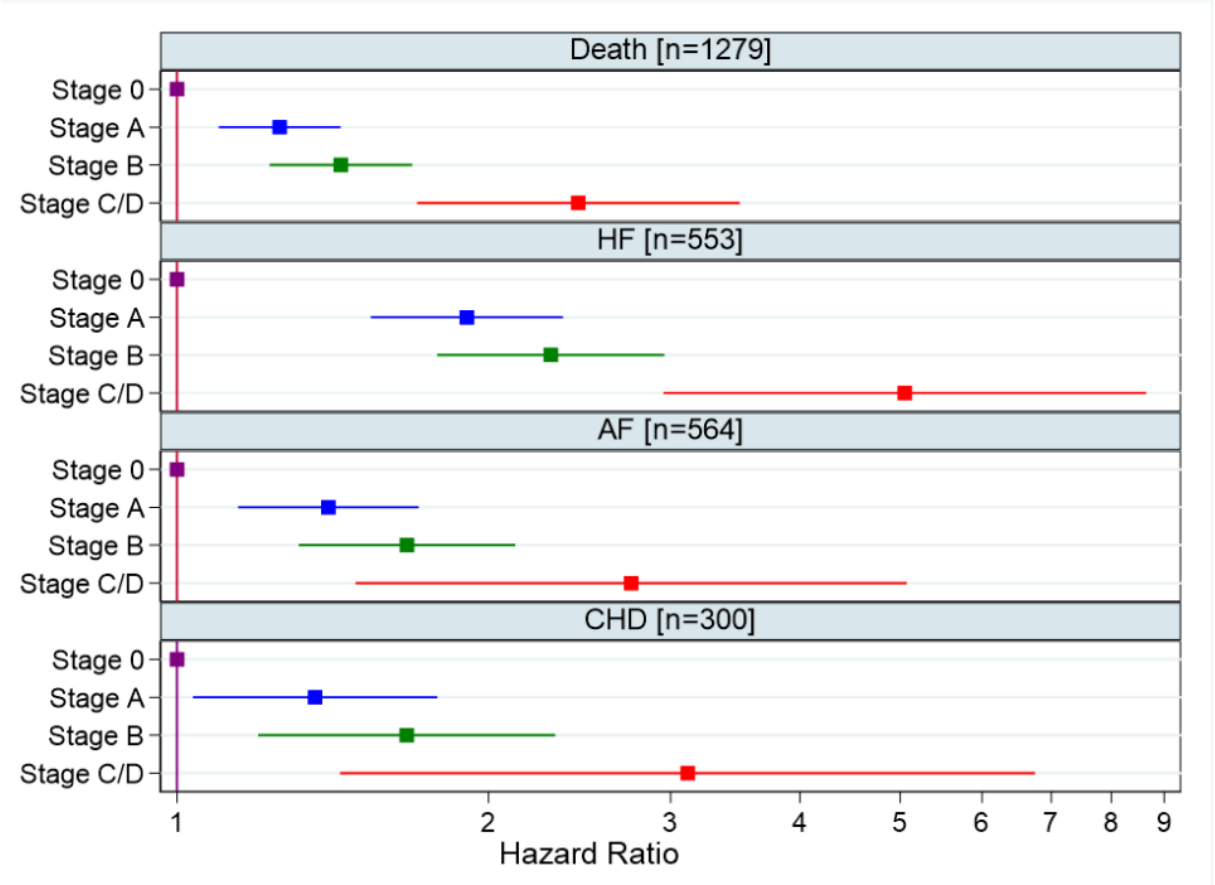

D
AS
AR
MS
MR
79%
15%
4%
0.9%
0.5%
76%
13%
11%
0.5%
84%
16%
0.2%
0.2%
57%
39%
4%
0.2%
0.2%
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Stage 0
Stage A
Stage B
Stage C/D
Replaced valve

Figure 3: Concentrations (median, IQR) of NT-proBNP (Panel A) and hs-TnT (Panel B) by VHD stage in ARIC at Visit 5.



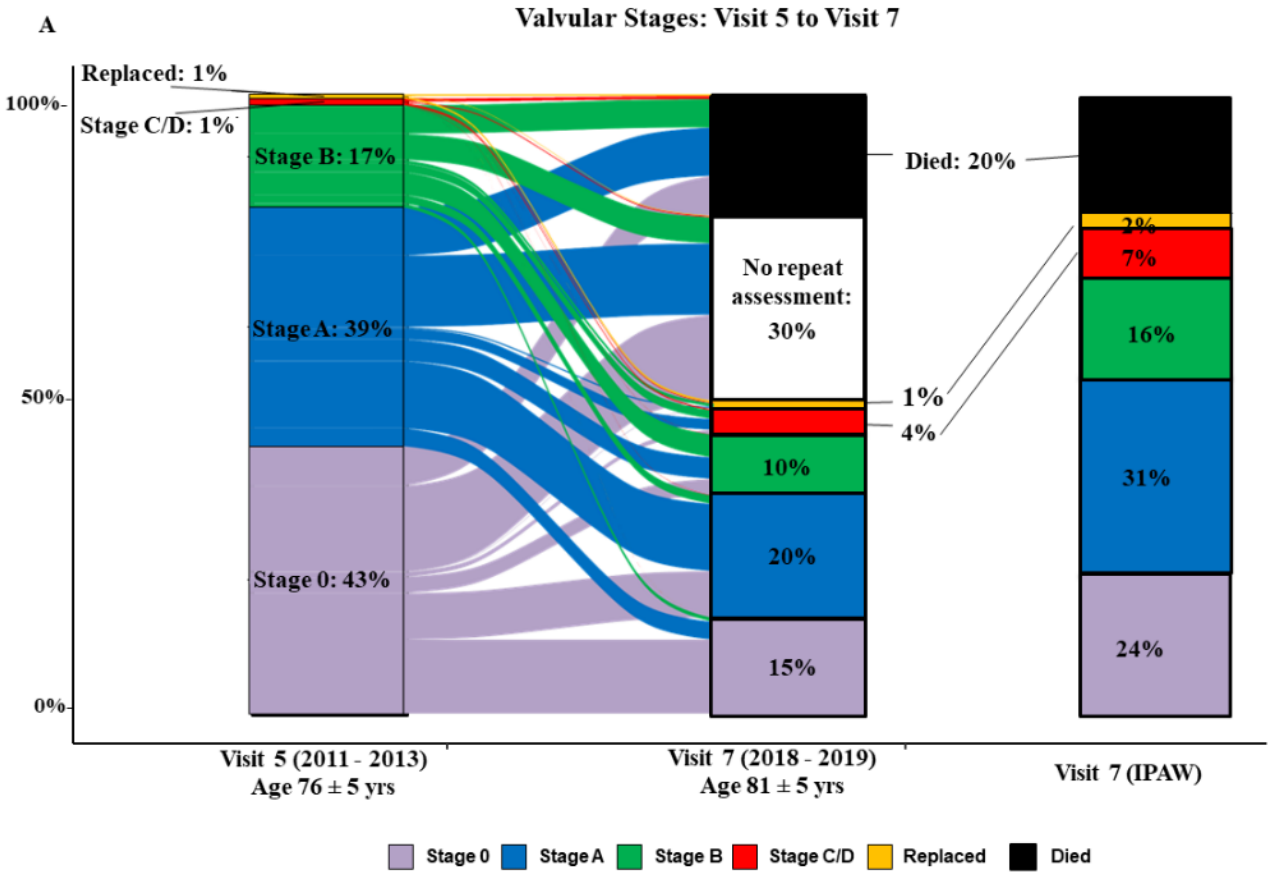
*p<0.05 compared to Stage 0; ^ p<0.05 compared to Stage A; ~ p<0.05 compared to Stage B.
Regression model was adjusted for age, sex, race, hypertension, diabetes, prior myocardial infarction, heart failure, body mass index, Field Center, and systolic blood pressure at Visit 5

Figure 4: Association of each VHD stage with incident CV events relative to VHD Stage free (Stage 0) for overall VHD.



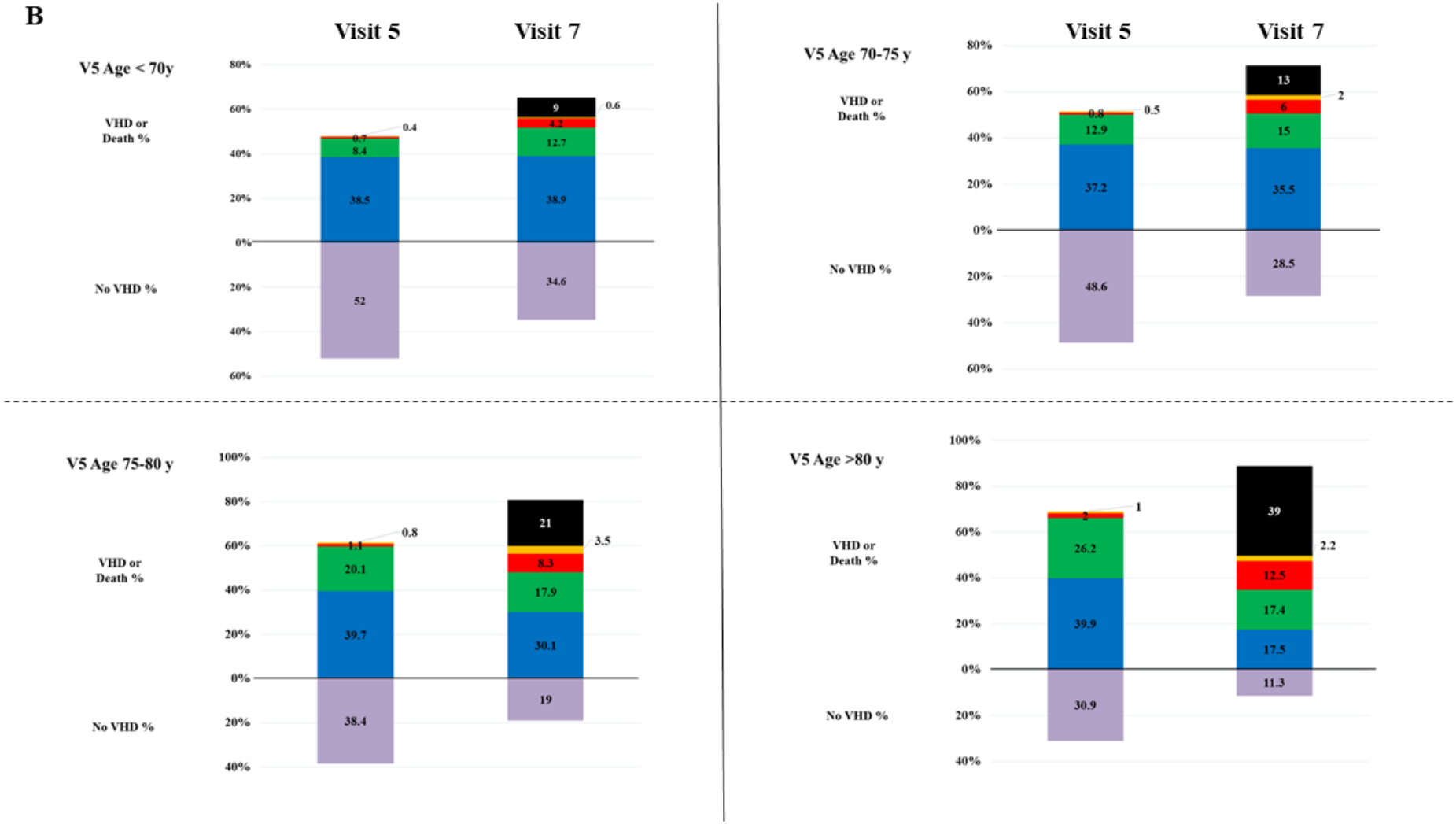
Forest plots demonstrate adjusted HR (95% CI) adjusted for age, sex, race, hypertension, diabetes, prior myocardial infarction, heart failure, body mass index, Field Center, and systolic blood pressure at Visit 5**.**

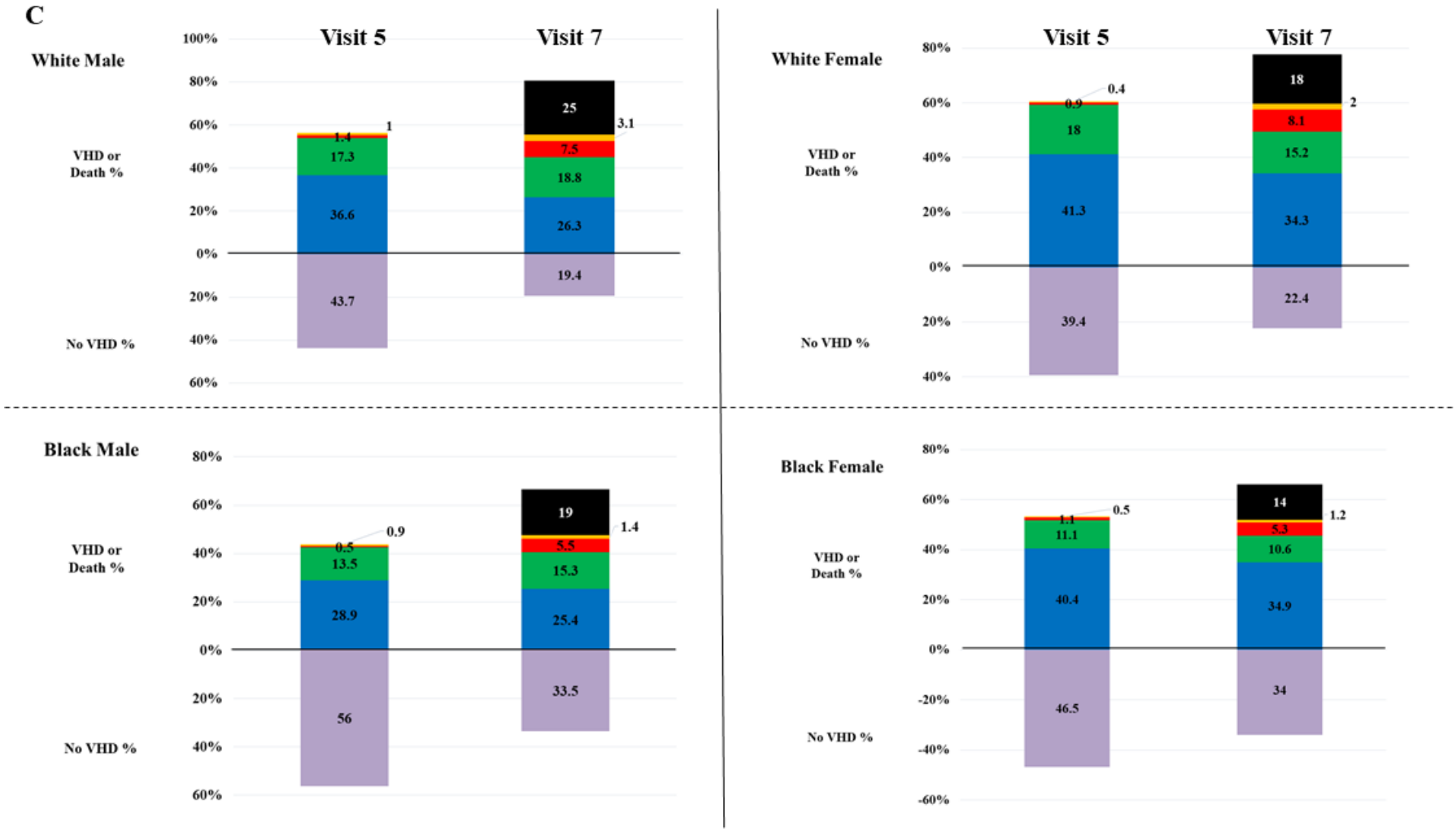
Figure 5: Transitions of VHD stages over 6.6 years from ARIC Visit 5 (mean age 76±5 years) to Visit 7 (mean age 81± 4 years) overall (Panel A), by age category (Panel B), and by race and gender groups (Panel C).



Sankey diagram in Panel A demonstrates the transition in VHD stage from Visit 5 to Visit 7 (left 2 columns). Right-most column demonstrates prevalence of VHD stages at Visit 7 using IPAW to account for Visit 7 non-attendance.

B


Visit 5
Visit 7
V5 Age < 70y
VHD or Death %
No VHD %
80%
60%
40%
20%
0%
20%
40%
60%
0.7
8.4
38.5
52
0.4
9
4.2
12.7
38.9
34.6
0.6
V5 Age 70-75 y
0.8
12.9
37.2
48.6
0.5
13
6
15
35.5
28.5
2
V5 Age 75-80 y
100%
1.1
20.1
39.7
38.4
0.8
21
8.3
17.9
30.1
19
3.5
V5 Age >80 y
2
26.2
39.9
30.9
1
39
12.5
17.4
17.5
11.3
2.2



For VHD transitions by age group (Panel B) and race and gender groups (Panel C), Visit 7 prevalence estimates are IPAW-adjusted. For VHD transitions by race and gender groups (Panel C), prevalence estimates are age adjusted.

Supplemental Materials
Operational definitions:

For the aortic valve (AV), Stage A (at risk) was defined as the presence of a bicuspid aortic valve or aortic sclerosis based on a peak aortic valve (AV) velocity of 1.5 – 2.0 m/sec. Stages B aortic stenosis (AS) was defined based on a peak AV velocity of 2.0 – 3.9 m/sec, while Stage C was determined based on a peak AV ≥4.0 m/sec, mean pressure gradient ≥ 40 mmHg or aortic valve area (AVA) <1 cm2 in case of absence of symptoms. Stage D definition was like Stage C but with symptoms of heart failure (HF), including dyspnea and exhaustion or the presence of anginal pain in their annual follow-up questionnaire closest to the visit date.

For aortic regurgitation (AR), Stage B (mild or moderate AR) and Stage C (severe AR) were based on a qualitative assessment of regurgitation severity based on color Doppler appearance in 4 standard views (parasternal long and short axis, and apical 5- and 3-chamber views). Stage D AR was defined as severe AR with symptoms of HF, including dyspnea and exhaustion.

For mitral stenosis (MS), Stage A was defined as the presence of rheumatic deformity or circumferential mitral annular calcification (MAC). To determine Stage B MS, we used qualitative assessment to detect mild MS in addition to the equivalent pressure half time and mitral valve area calculated from the deceleration time of mitral inflow. (1) Calculated mitral valve area ≤ 1.5 cm^2 was used to define Stages C and D.

Mitral regurgitation (MR) was quantified based on the greatest MR jet area-to-left atrial area ratio from the apical 4- and 2-chamber view as follows: 5- 20% - mild; 20-40% - moderate; ≥40% - severe. The MR Stage was increased by 1 grade if an eccentric regurgitant jet was present. Stage A mitral regurgitation (MR) was defined as the

presence of MAC, mitral valve prolapse or mild MR, Stage B as moderate MR, Stage C as severe MR without symptoms of HF, and Stage D as severe MR with HF symptoms. In our analyses, we combined severe VHD stages (i.e., Stage C and D) in one group (n=66) due to limited numbers in those Stages.

ICD Codes used to detect VHD related hospitalizations and deaths

The diagnostic ICD codes that were checked in the ARIC cohort surveillance data-set included: (ICD-9 codes) 424.0, 394.0, 396.0 for mitral valve disease (2) or 424.1 for aortic valve disease (3); and (ICD-10 codes) I34.0 for MR, I34.2 for MS, 135.1 for AR, (4) or I35.0, I35.2 for AS(3).

While the procedures ICD codes included : (ICD-9-PCS codes) 35.11, 35.12 (5), 35.21, 35.22 (6), 35.23, 35.24 (7), 35.05 or 35.06(8); or (ICD-10-PCS codes) 02NFxx, 027Fxx, 02QFxx, 02RFxx, , 02RF0xx, 02RF4xx, X2RF0xx, 02NGxx, 027Gxx, 02QGxx, 02VGxx, 02RGxx, 027Gxx (5), 02RF37H, 02RF38H, 02RF3J, 02RF3KH, 02RF37Z, 02RF38Z, 02RF3JZ or 02RF3KZ. (8)

Supplementary References

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Supplement Table 1: Event rates and HRs for incident events post-V5 by VHD Stage after incorporating IPAW for Visit 5 attendance

		Events	Rate per 100 PY	HR (Model 1)	P-value	HR (Model 2)	P-value
Death	Stage 0	795	2.8 (2.5-3.1)	Reference group			
	Stage A	1060	4 (3.7-4.4)	1.3(1.2-1.5)	**<0.001**	1.3(1.1-1.5)	**<0.001**
	Stage B	625	5.5 (4.9-6.1)	1.5(1.3-1.8)	**<0.001**	1.4(1.2-1.7)	**<0.001**
	Stage C/D	77	10.8 (8.5-13.9)	3(2.3- 4)	**<0.001**	2.5(1.9-3.4)	**<0.001**
HF	Stage 0	288	1.1(0.9-1.3)	Reference group			
	Stage A	477	2(1.8-2.3)	1.8(1.4-2.2)	**<0.001**	1.9(1.5-2.3)	**<0.001**
	Stage B	269	2.7(2.3-3.3)	2.2(1.7-2.8)	**<0.001**	2.2(1.7-2.9)	**<0.001**
	Stage C/D	28	5.5 (3.4-9.4)	4.5(2.6-7.8)	**<0.001**	4.5(2.5-8.1)	**<0.001**
AF	Stage 0	333	1.6(1.3-1.8)	Reference group			
	Stage A	446	2.4(2.1-2.8)	1.5(1.2-1.8)	**<0.001**	1.4(1.1-1.7)	**0.002**
	Stage B	246	3.3(2.8-4)	1.8(1.4-2.3)	**<0.001**	1.7(1.3-2.2)	**<0.001**
	Stage C/D	24	6.2(3.5-11.7)	3.3(1.8-6.1)	**<0.001**	3.1(1.6-5.9)	**0.001**
CHD	Stage 0	176	0.7(0.6-0.9)	Reference group			
	Stage A	234	1.1(0.9-1.3)	1.5(1.1-2)	**0.004**	1.5(1.1-2)	**0.008**
	Stage B	123	1.3(1.0-1.8)	1.7(1.2-2.4)	**0.001**	1.7(1.2-2.4)	**0.003**
	Stage C/D	16	3.3(1.6-8.3)	4.2(1.9-9.4)	**<0.001**	3.9(1.7-9.0)	**0.002**
Stroke	Stage 0	176	0.6(0.5-0.8)	Reference group			
	Stage A	177	0.7(0.6-0.9)	1.1(0.8-1.4)	0.642	1.1(0.8-1.5)	0.550
	Stage B	101	0.9(0.7-1.3)	1.3(0.9-1.9)	0.141	1.3(0.9-1.8)	0.224
	Stage C/D	4	0.5(0.1-5.4)	0.8(0.2-3.1)	0.723	0.4(0.1-2.7)	0.360

Model 1: adjusted for age, sex, and race
Model 2: adjusted for age, sex, race, Field Center, hypertension, diabetes, prior myocardial infarction, heart failure, body mass index, and systolic blood pressure**.**

Supplement Table 2: the association of each endpoint with individual lesion Stages A and B

A- Death

	N	Events	Rate per 100 PY	HR (Model 1)	P-value	HR (Model 2)	P-value
AS	**Total N with a Stage less than Stage C =6025**						
Stage 0	4,840	896 (19%)	2.8(2.6-3.0)	Reference group			
Stage A	937	256(27%)	4.2(3.7-4.8)	1.4(1.2-1.6)	**<0.001**	1.4(1.2-1.6)	**<0.001**
Stage B	248	92(37%)	6.0(4.9-7.4)	1.7(1.3-2.1)	**<0.001**	1.6(1.3-2.0)	**<0.001**
AR	**Total N with a Stage less than Stage C =6078**						
Stage 0	4,611	874(19%)	2.9(2.7-3.1)	Reference group			
Stage A	818	217(27%)	4.1(3.6-4.7)	1.3(1.1-1.5)	<0.001	1.4(1.2-1.6)	<0.001
Stage B	649	188(29%)	4.5(3.9-5.2)	1.2(1.0-1.4)	0.031	1.1(1.0-1.4)	0.104
MS	**Total N with a Stage less than Stage C =6077**						
Stage 0	5,087	1018(20%)	3.0(2.9-3.2)	Reference group			
Stage A	980	253(26%)	4.0(3.6-4.6)	1.2(1.0-1.4)	0.009	1.1(1.0-1.3)	0.107
Stage B	10	8(80%)	17.2(8.5-34.3)	4.5(2.2-9)	<0.001	4.2(2.1-8.5)	<0.001
MR	**Total N with a Stage less than Stage C =6066**						
Stage 0	3,476	628 (18%)	2.7(2.5-3.0)	Reference group			
Stage A	2,347	560 (24%)	3.7(3.4-4.0)	1.2(1.1-1.4)	0.001	1.2(1.0-1.3)	0.014
Stage B	243	88 (36%)	5.8(4.7-7.2)	1.8(1.4-2.2)	<0.001	1.7(1.3-2.1)	<0.001

Model 1: adjusted for age, sex, and race

Model 2: adjusted for age, sex, race, Field Center, hypertension, diabetes, prior myocardial infarction, heart failure, body mass index, and systolic blood pressure**.**

Supplement Table 2: the association of each endpoint with individual lesion Stages A and B

B- HF

	N	Events	Rate per 100 PY	HR (Model 1)	P-value	HR (Model 2)	P-value
AS	**Total N with a Stage less than Stage C** = 5,487						
Stage 0	4,447	379(9%)	1.3(1.2-1.4)	Reference group			
Stage A	830	105(13%)	1.9(1.6-2.3)	1.4(1.1-1.8)	0.002	1.4(1.1-1.8)	0.003
Stage B	210	54(26%)	4.2(3.2-5.5)	2.7(2.0-3.6)	<0.001	2.5(1.8-3.3)	<0.001
AR	**Total N with a Stage less than Stage C** =5,527						
Stage 0	4,243	388(9%)	1.4(1.2-1.5)	Reference group			
Stage A	728	93(13%)	1.9(1.6-2.4)	1.3(1.1-1.7)	0.015	1.3(1.0-1.7)	0.024
Stage B	556	72(13)	1.9(1.5-2.4)	1.2(0.9-1.5)	0.276	1.2(0.9-1.6)	0.136
MS	**Total N with a Stage less than Stage C** =5,526						
Stage 0	4,663	424(9%)	1.4(1.2-1.5)				
Stage A	857	126(15%)	2.3(1.9-2.7)	1.5(1.3-1.9)	<0.001	1.5(1.2-1.9)	<0.001
Stage B	6	3(30%)	10.3(3.3-31.8)	6.1(1.9-18.9)	0.002	7.2(2.3-22.8)	0.001
MR	**Total N with a Stage less than Stage C** = 5,515						
Stage 0	3,254	238 (7%)	1.1(1.0-1.2)	Reference group			
Stage A	2,063	277(13%)	2.0(1.8-2.3)	1.7(1.5-2.1)	<0.001	1.8(1.5-2.2)	<0.001
Stage B	198	37(19%)	3.0(2.2-4.1)	2.5(1.7-3.5)	<0.001	2.6(1.8-3.8)	<0.001

Model 1: adjusted for age, sex, and race

Model 2: adjusted for age, sex, race, Field Center, hypertension, diabetes, prior myocardial infarction, heart failure, body mass index, and systolic blood pressure**.**

Supplement Table 2: the association of each endpoint with individual lesion Stages A and B

C- AF

	N	Events	Rate per 100 PY	HR (Model 1)	P-value	HR (Model 2)	P-value
AS	**Total N with a Stage less than Stage C** = 5,487						
Stage 0	4,447	407(9%)	1.8(1.6-2.0)	Reference group			
Stage A	830	105(13%)	2.5(2.1-3.0)	1.3(1.1-1.6)	0.011	1.3(1.0-1.6)	0.019
Stage B	210	41(20%)	4.1(3.0-5.5)	1.8(1.3-2.5)	<0.001	1.4(1.0-2.1)	0.040
AR	**Total N with a Stage less than Stage C** = 5,527						
Stage 0	4,243	402(9%)	1.8(1.7-2.0)	Reference group			
Stage A	728	86(12%)	2.3(1.9-2.9)	1.2(0.9-1.5)	0.138	1.2(0.9-1.5)	0.173
Stage B	556	76(14%)	2.8(2.2-3.5)	1.2(1.0-1.6)	0.084	1.3(1.0-1.7)	0.041
MS	**Total N with a Stage less than Stage C** =5,526						
Stage 0	4,663	461(10%)	1.9(1.8-2.1)				
Stage A	857	101(12%	2.4(1.9-2.9)	1.1(0.9-1.4)	0.224	1.1(0.8-1.3)	0.602
Stage B	6	2(33%)	8.8(2.2-35.3)	3.6(0.9-14.7)	0.069	3.6(0.9-14.8)	0.071
MR	**Total N with a Stage less than Stage C** = 5,515						
Stage 0	3,254	274 (8%)	1.6(1.4-1.8)	Reference group			
Stage A	2,063	255(12%)	2.5(2.2-2.8)	1.4(1.2-1.7)	<0.001	1.4(1.1-1.7)	0.001
Stage B	198	34(17%)	3.6(2.6-5.0)	2.0(1.4-2.8)	<0.001	2.1(1.5-3.1)	<0.001

Model 1: adjusted for age, sex, and race

Model 2: adjusted for age, sex, race, Field Center, hypertension, diabetes, prior myocardial infarction, heart failure, body mass index, and systolic blood pressure**.**

Supplement Table 2: the association of each endpoint with individual lesion Stages A and B

D- CHD

	N	Events	Rate per 100 PY	HR (Model 1)	P-value	HR (Model 2)	P-value
AS	**Total N with a Stage less than Stage C =5231**						
Stage 0	4,242	217(5%)	0.8(0.7-0.9)	Reference group			
Stage A	798	53(7%)	1.0(0.8-1.4)	1.3(0.9-1.7)	0.13	1.2(0.9-1.6)	0.263
Stage B	191	23(12%)	2.0(1.3-3.0)	2.3(1.5-3.5)	<0.001	2.0(1.3-3.2)	0.002
AR	**Total N with a Stage less than Stage C =5270**						
Stage 0	4,023	217(5%)	0.8(0.7-1.0)	Reference group			
Stage A	703	50(7%)	1.1(0.8-1.5)	1.3(0.9-1.7)	0.115	1.2(0.9-1.7)	0.185
Stage B	544	33(6%)	1.0(0.7-1.4)	1.0(0.7-1.5)	0.969	1.1(0.8-1.6)	0.544
MS	**Total N with a Stage less than Stage C =5270**						
Stage 0	4,452	237(5%)	0.8(0.7-0.9)				
Stage A	812	63(8%)	1.3(1.0-1.6)	1.5(1.1-2.0)	0.006	1.5(1.1-2.0)	0.009
Stage B	6	0					
MR	**Total N with a Stage less than Stage C =5259**						
Stage 0	3,088	166 (5%)	0.8(0.7-1.0)	Reference group			
Stage A	1,964	121(6%)	1.0(0.8-1.2)	1.2(0.9-1.5)	0.208	1.1(0.9-1.5)	0.288
Stage B	207	13(6%)	1.0(0.6-1.8)	1.2(0.7-2.1)	0.522	1.1(0.6-2.1)	0.709

Model 1: adjusted for age, sex, and race

Model 2: adjusted for age, sex, race, Field Center, hypertension, diabetes, prior myocardial infarction, heart failure, body mass index, and systolic blood pressure

Supplement Table 2: the association of each endpoint with individual lesion Stages A and B

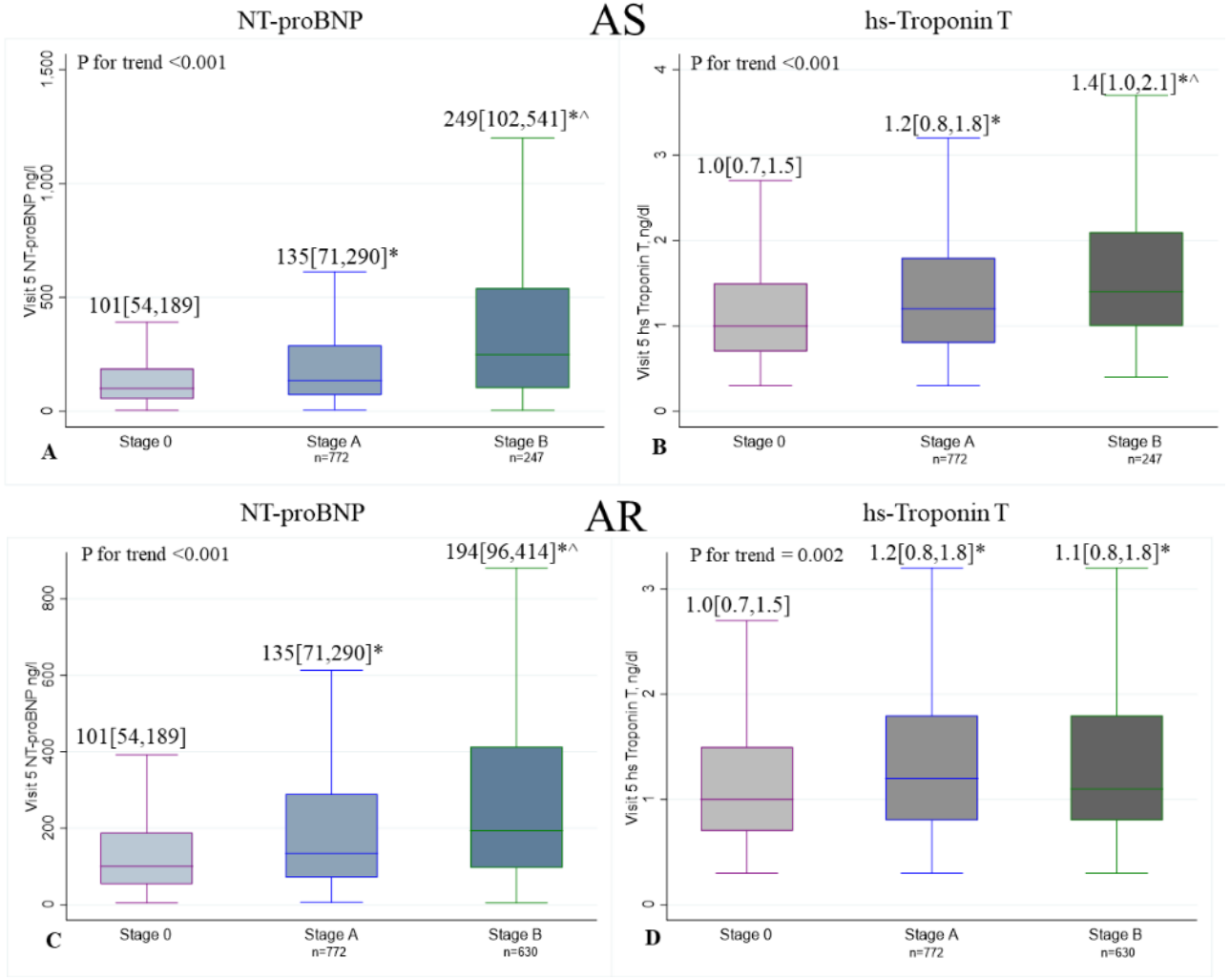
D - Stroke

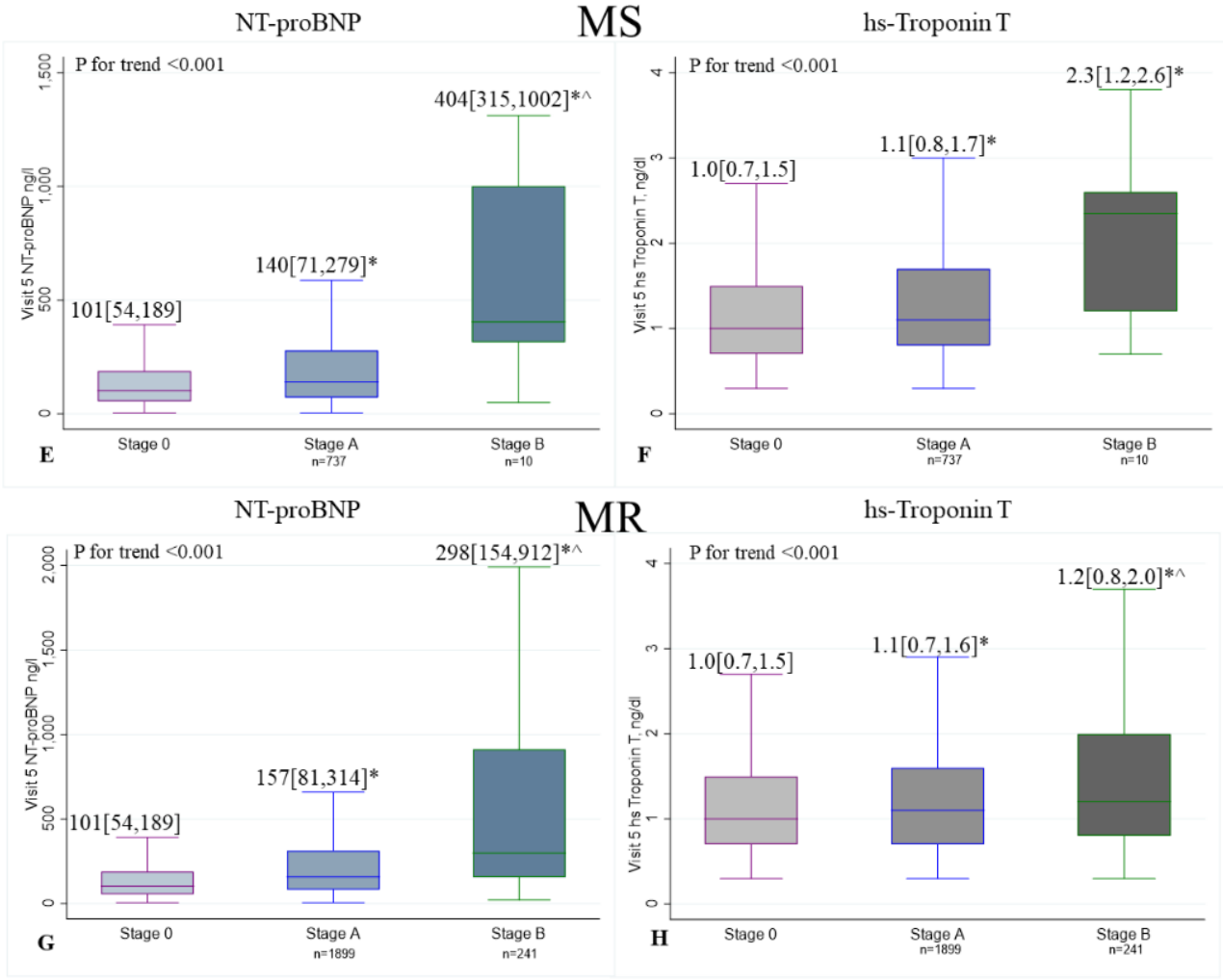
	N	Events	Rate per 100 PY	HR (Model 1)	P-value	HR (Model 2)	P-value
AS	**Total N with a Stage less than Stage C =5835**						
Stage 0	4,704	192(4%)	0.6(0.5-0.7)	Reference group			
Stage A	903	40(4%)	0.7(0.5-1.0)	1.1(0.8-1.5)	0.719	1.1(0.8-1.6)	0.583
Stage B	228	16(7%)	1.2(0.7-1.9)	1.6(1.0-1.8)	0.062	1.6(0.9-2.7)	0.097
AR	**Total N with a Stage less than Stage C = 5886**						
Stage 0	4,472	185(4%)	0.6(0.6-0.7)	Reference group			
Stage A	786	34(4%)	0.7(0.5-1.0)	1.0(0.7-1.5)	0.884	1.1(0.7-1.6)	0.700
Stage B	628	31(5%)	0.8(0.6-1.1)	1.0(0.7-1.5)	0.815	1.1(0.7-1.6)	0.721
MS	**Total N with a Stage less than Stage C = 5885**						
Stage 0	4,944	211(4%)	0.7(0.6-0.8)				
Stage A	931	36(4%)	0.6(0.4-0.8)	0.9(0.6-1.2)	0.467	0.9(0.6-1.3)	0.498
Stage B	10	3(30%)	6.5(2.1-20.1)	8.1(2.6-25.5)	<0.001	10.8(3.4-34.5)	<0.001
MR	**Total N with a Stage less than Stage C = 5874**						
Stage 0	3,377	130(4%)	0.6(0.5-0.7)	Reference group			
Stage A	2,262	110(5%)	0.8(0.6-0.9)	1.2(0.9-1.6)	0.144	1.2(0.9-1.6)	0.127
Stage B	235	10(4%)	0.7(0.4-1.3)	1.1(0.6-2.0)	0.836	1.1(0.5-2.1)	0.868

Model 1: adjusted for age, sex, and race

Model 2: adjusted for age, sex, race, Field Center, hypertension, diabetes, prior myocardial infarction, heart failure, body mass index, and systolic blood pressure**.**

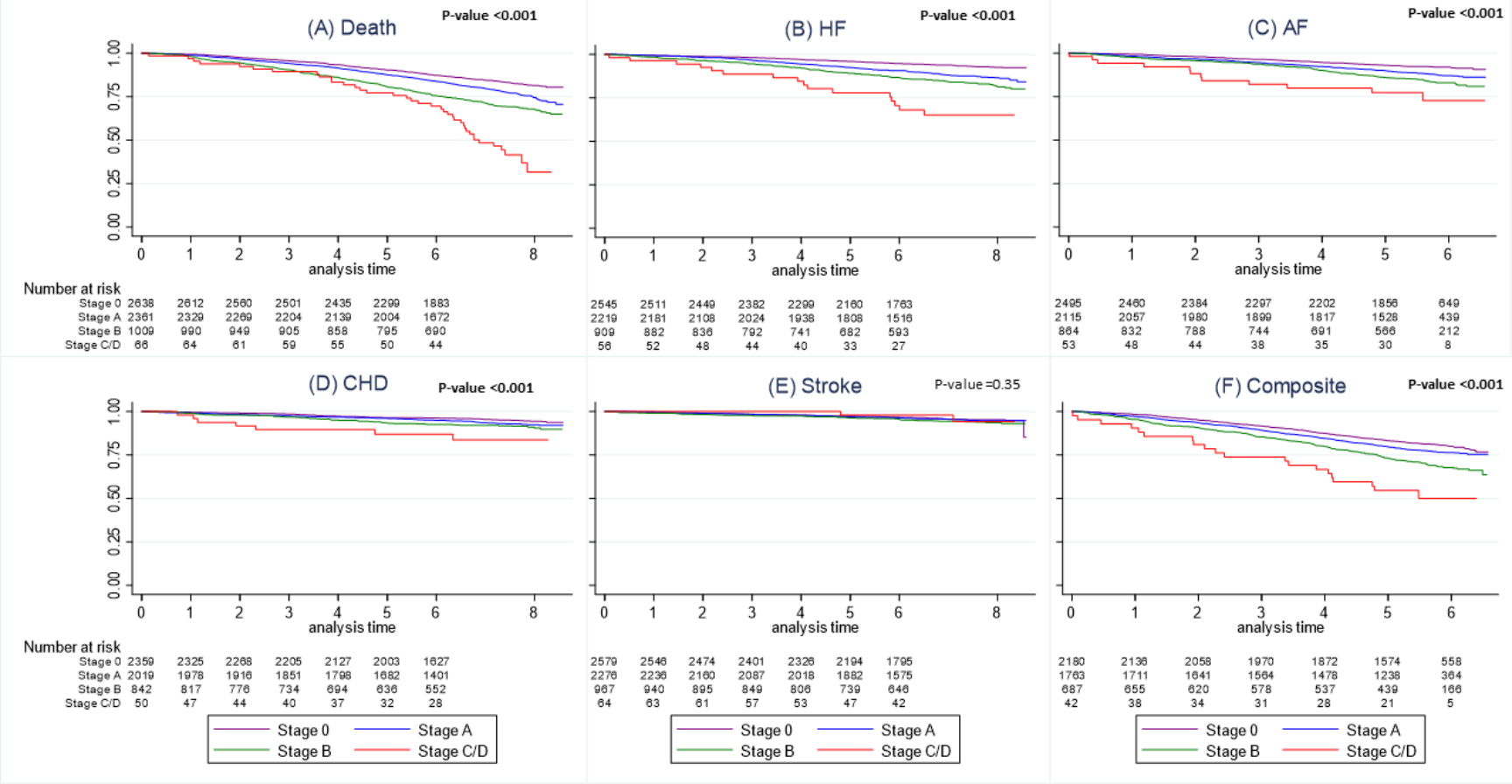
Supplement Figure-1: Concentrations (median, IQR) of NT-proBNP (A,C,E,G) and hs-TnT (B,D,F,H) by individual lesion Stages A and B after excluding those who would be upgraded due to another lesion



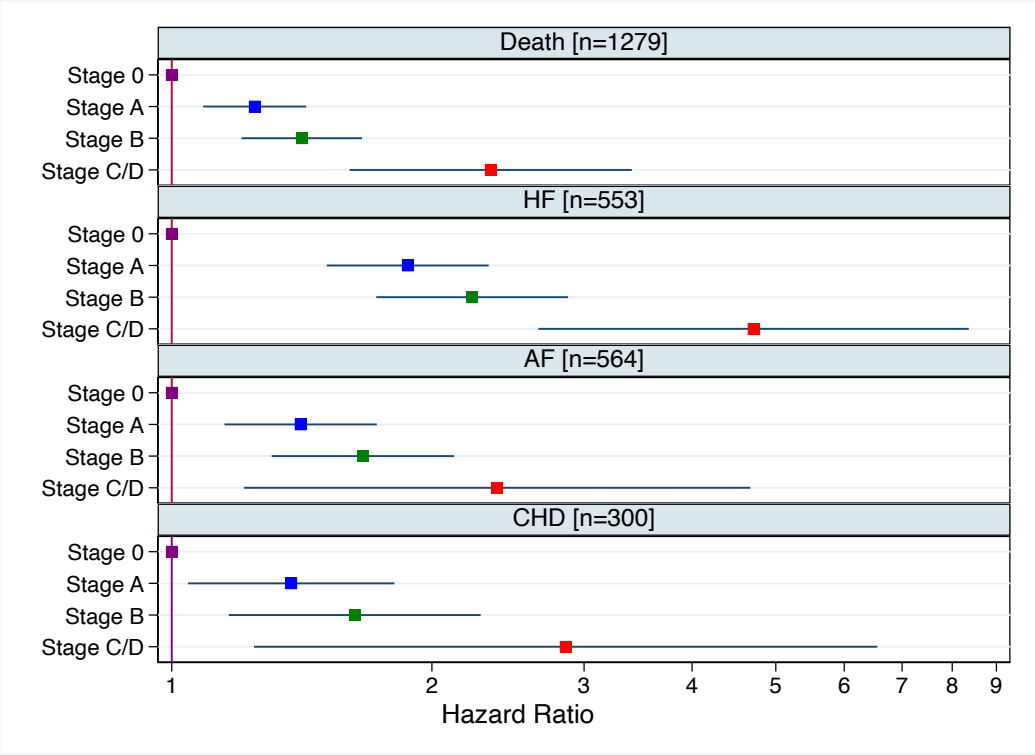


*p<0.05 compared to Stage 0; ^ p<0.05 compared to Stage A; ~ p<0.05 compared to Stage B. Regression model was adjusted for age, sex, race, hypertension, diabetes, prior myocardial infarction, heart failure, body mass index, Field Center, and systolic blood pressure at Visit 5

Supplement Figure-2: VHD stages and incident events: Kaplan-Meier curves

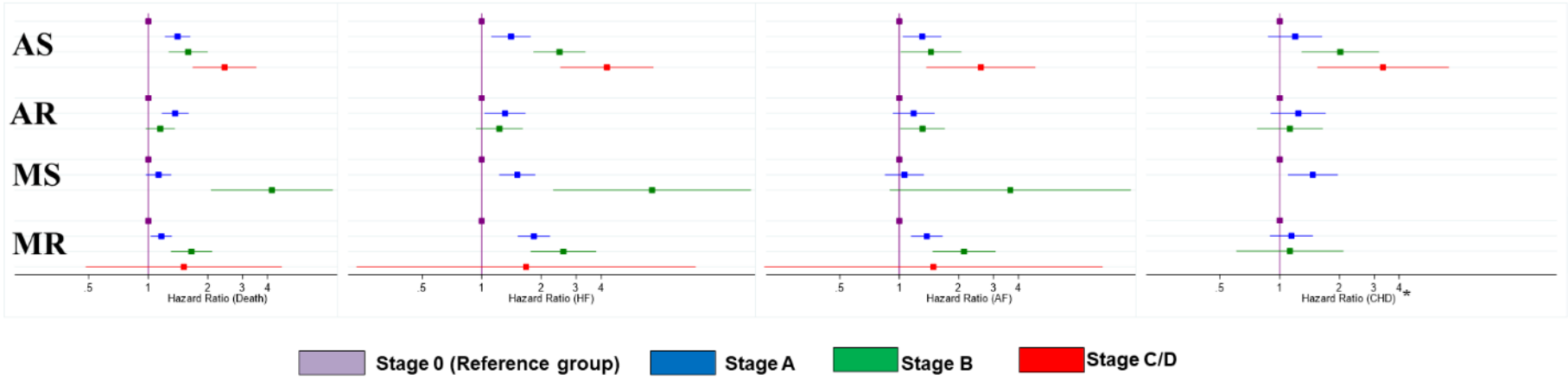


Supplement Figure-3: Association of overall VHD Stages with incident CV events relative to VHD Stage free (Stage 0) after adding eGFR and LDL to model2 as a sensitivity analysis



Forest plots demonstrate adjusted HR (95% CI), adjusted for age, sex, race, hypertension, diabetes, prior myocardial infarction, heart failure, body mass index, Field Center, systolic blood pressure, estimated glomerular filtration rate and low-density lipoprotein at Visit 5.

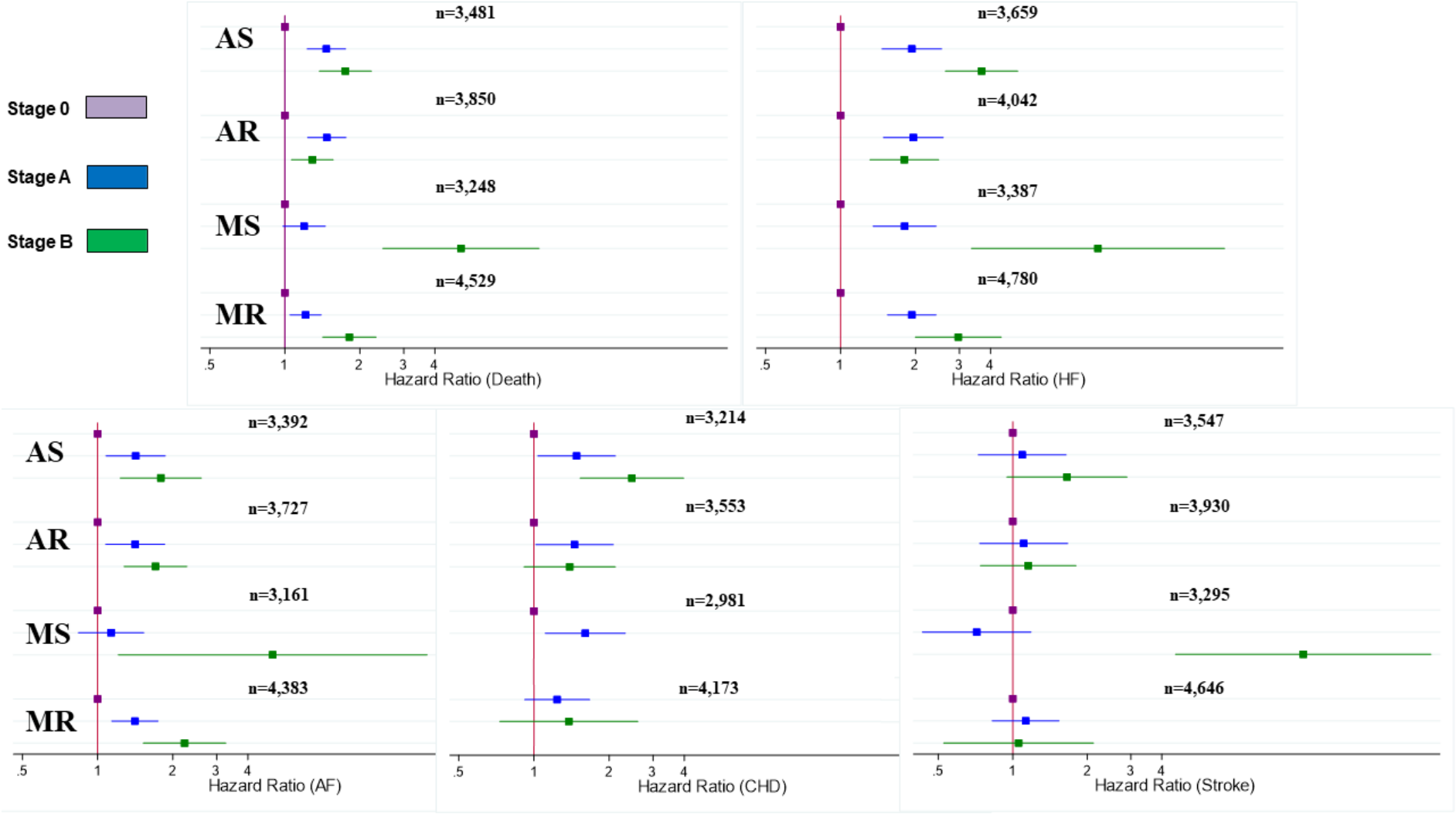
Supplement Figure-4: Association of each VHD Stage with incident CV events relative to VHD Stage free (Stage 0) by valve lesion



Forest plots demonstrate adjusted HR (95% CI), adjusted for age, sex, race, hypertension, diabetes, prior myocardial infarction, heart failure, body mass index, Field Center, and systolic blood pressure at Visit 5**.**

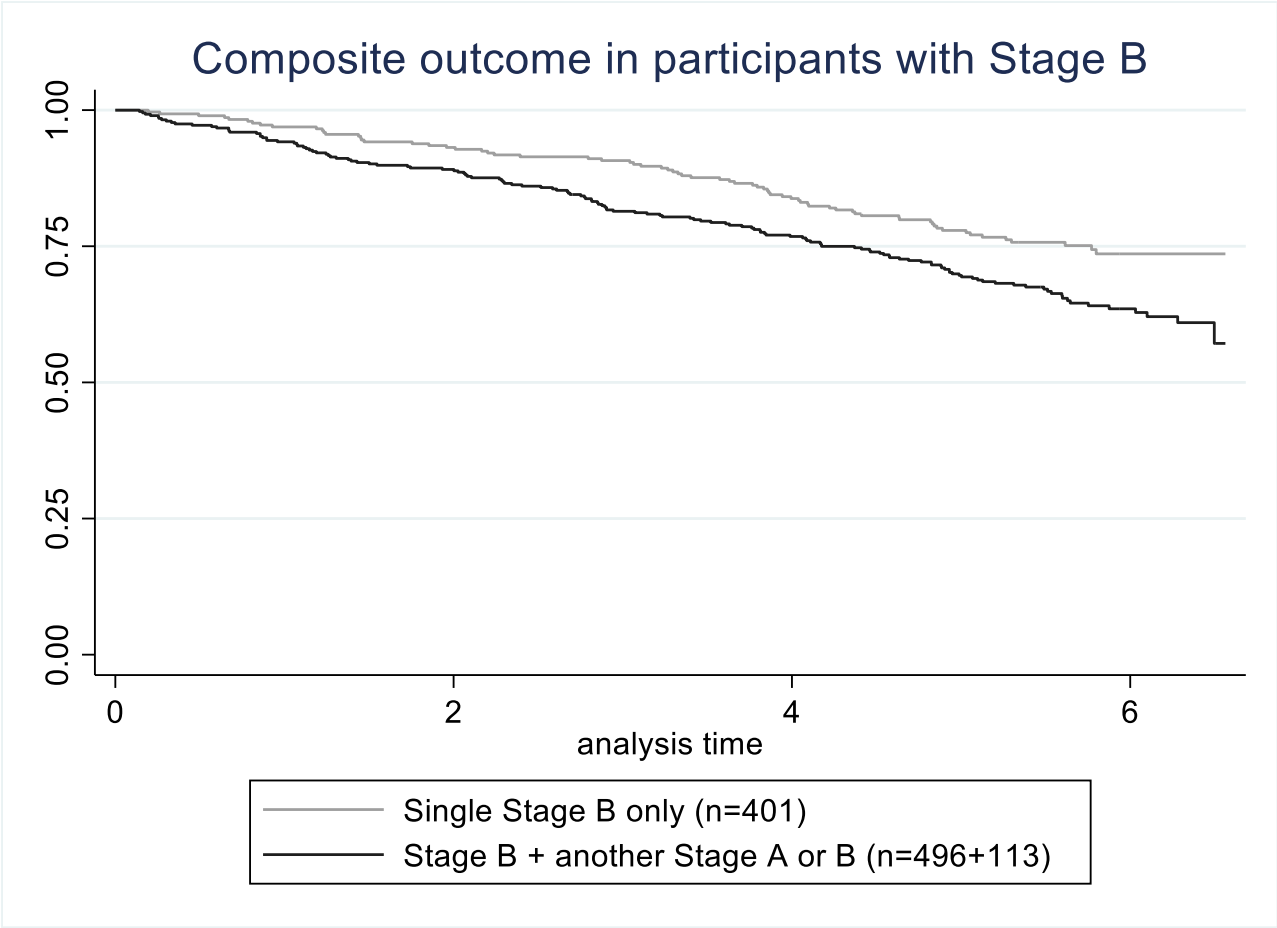
***There were no coronary heart disease events in Stage B MS and Stage C/D MR

Supplement Figure-5: sensitivity analysis: the association (HR and 95% CI) of each endpoint with individual lesion Stages A and B after excluding those who would be upgraded due to another lesion

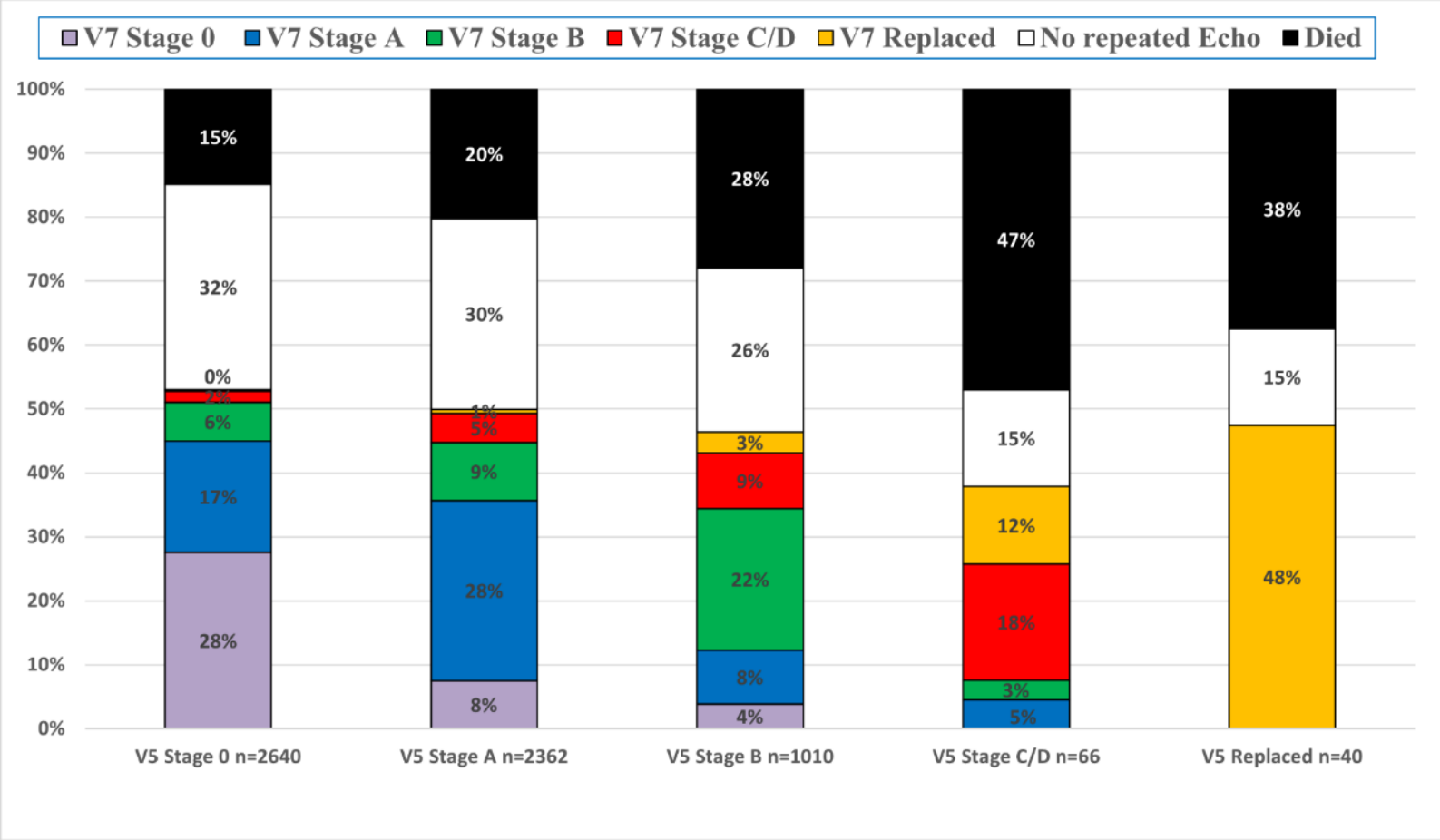


HRs are adjusted for (model 2 covariates) age, sex, race, Field Center, hypertension, diabetes, prior myocardial infarction, heart failure, body mass index, and systolic blood pressure**.**

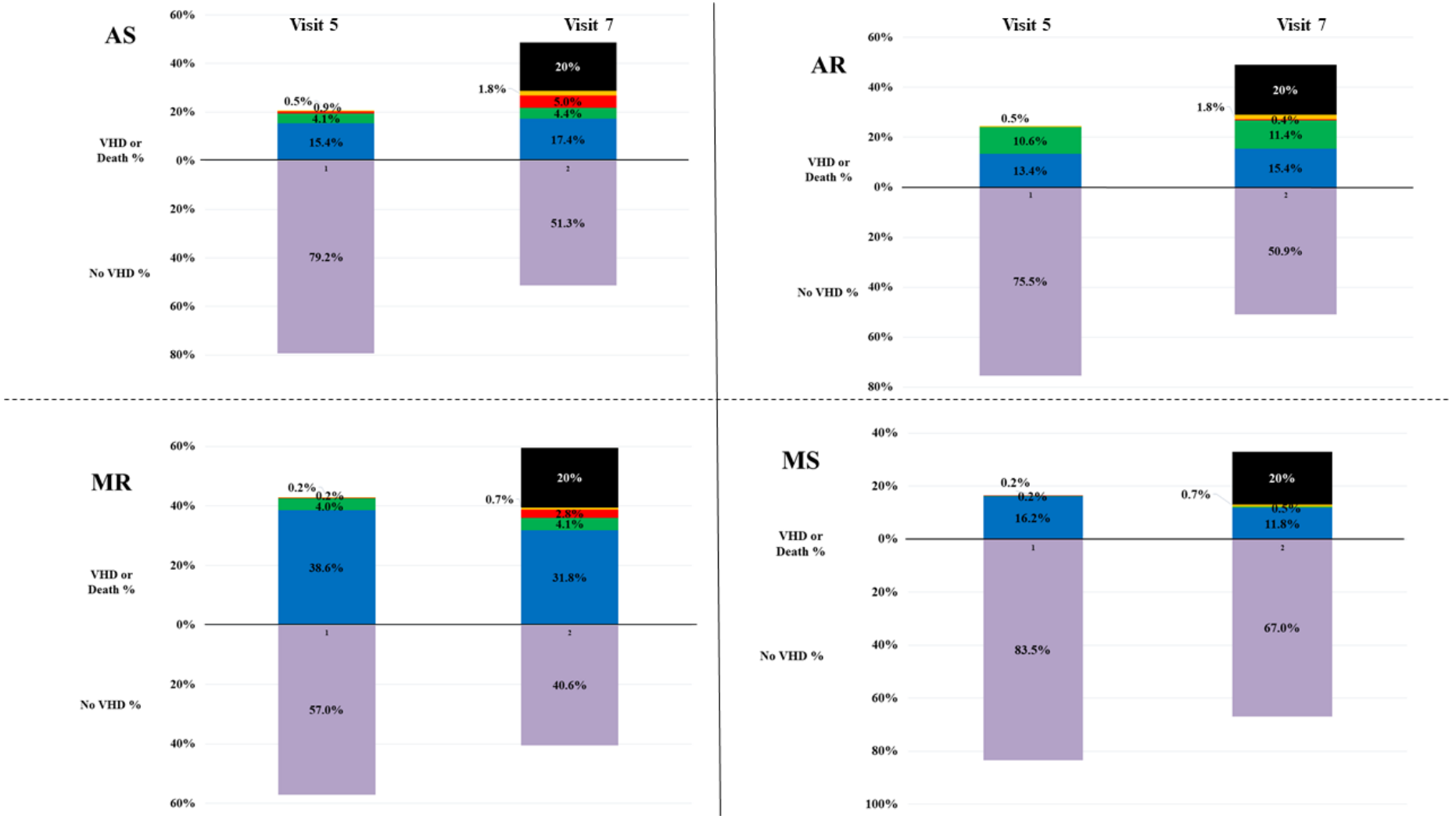
Supplement Figure 6: The Kapan -Meier curves showing the association of the number of VHD lesions with the composite outcome among the participants who had overall B.



Supplement Figure 7: The transition of the overall Visit 5 VHD stages into VHD stages at Visit 7, censoring or death (presented as percentages.



Supplement Figure 8: The transition of Visit 5 VHD stages into VHD stages at Visit 7 for each valvular lesion (after incorporating IPAW for alive participants who do not have repeat echo)**.**



Manuscript 2

Proteomic Markers of Aortic Stenosis: the Atherosclerosis Risk in Communities Study

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Abstract

Importance: Despite the increasing prevalence of aortic stenosis (AS), little is known regarding circulating biomarkers predictive of valve stenosis development.

Objective: Identify circulating biomarkers predictive of aortic valve (AV) stenosis.

Design, Setting, and Participants: Cross-sectional, time-to-event, and longitudinal analyses using the data from participants who had aptamer-based proteomics measurements from two separate visits in the Atherosclerosis Risk in Community (ARIC) prospective community-based cohort study and followed up till the end of 2019.

Exposures: Plasma proteomics measured using the SOMAscan aptamer-affinity assay (n=4,877 aptamers; Somalogic Inc., Boulder, CO) at study Visits 3 (V3) and 5 (V5).

Main Outcomes and Measures: Outcomes included measures of AV hemodynamics including peak velocity, dimensionless index, and aortic valve area assessed by protocol echocardiography at V5 and Visit 7 (V7); incident AS-associated hospitalization or AV replacement after V3 ascertained using hospitalization ICD codes; and AV calcification by cardiac-gated non-contrast computerized tomography (CT) scan at V7.

Results: At V5 (n=4,899 with mean age 76 ± 5 years; 43% male; 18% Black adults), 917 proteins were cross-sectionally associated with AV peak velocity at FDR p <0.05 in multivariable linear regression models adjusting for demographics and cardiovascular co-morbidities. At V3, (n=11,430 with mean age 60 ± 6 years; 46% male; 21% Black), 72 of these 917 were associated with risk of incident AS-related hospitalization post-V3 (median follow-up of 22.2 [IQR 14.4 – 24.8] years, n=912 events). Of these 72 proteins, 52 were also cross-sectionally associated with the AV dimensionless index at V5, 14 associated with lower hemodynamic AS severity, and the remainder associated with greater AS severity. Of these, 38 proteins assessed at V5 were significantly associated with aortic leaflet calcification by CT at V7 (n=1802) and two were associated with the change in AV peak velocity between V5 and V7 (n=2,314; mean time interval 6.3 ± 0.6 years). MMP12 emerged as robustly associated with all study outcomes including change in AV peak velocity from V5 and V7 and magnitude of AV calcification at V7 (beta-coefficient 2.6 (1.1, 4.2) p=9.2e-04, OR1.2 (1.2, 1.3) p=1.1e-16, respectively). Pathway analysis suggested a potential role of interferon-gamma as an upstream regulator for AS.

Conclusions and Relevance: We identified 38 circulating proteins with robust associations with AV hemodynamics, calcification, and risk of incident AV-related hospitalizations. Higher MMP12 values demonstrated particularly robust and consistent associations with worse AV hemodynamics, progression in AV over time, magnitude of AV calcification, and risk of incident AV events. These findings highlight a potential novel biomarker for AS risk, and a novel putative targetable pathway to prevent AS progression.

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Role of Funder

The funder had no role design and conduct of this study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Access to Data and Data Analysis

Dr AM Shah had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Disclosures

Dr Shah reports consulting fees from Philips Ultrasound and research funds from Novartis through Brigham and Women's Hospital. Dr. Skali reports consulting fees from Astellas Inc. and research support from ABT Associates.

Introduction

Aortic stenosis (AS) is the most common clinically significant valvular heart disease in the United States, and its prevalence increases with age. (1) Calcific aortic valve disease is the leading cause of AS and is characterized by progression from leaflet thickening and calcification (aortic sclerosis) to significant hemodynamic stenosis. (2) While the prevalence of severe AS among persons > 75 years of age is approximately 3.4 %, the prevalence of mild AS and aortic sclerosis is appreciably higher (34.6%). (3)The prevalence of significant AS – and the number of persons eligible for AV intervention – is therefore expected to increase as average life expectancy increases in Western countries. (4) Common atherosclerotic risk factors, including hypertension, diabetes, and dyslipidemia, are independently associated with severe AS in older populations.

(5) However, atherosclerosis-modifying therapies such as statin therapy have not proven effective at reducing AS progression, suggesting at least partially distinct mechanisms.(6-8) No therapy currently exists to prevent the progression of aortic calcification and stenosis. (9-11) Furthermore, beyond the existing measures of AS severity, few biomarkers exist to predict an individual's risk of progressive AS. Both N-terminal pro-brain natriuretic peptide (NT-proBNP) and high-sensitivity troponin (hsTn) are prognostic of clinical outcomes in AS, but largely reflect myocardial wall stress and injury in response to AS as opposed to underlying mechanistic processes driving calcific AV disease and have limited utility in predicting AS progression (12-16)

Several mechanistic pathways have been related to aortic calcification. Data from animal models (17-20) and explanted human AV tissue (21-23)implicate inflammation, neurohormonal activation, lipid oxidization, and biomineralization in progressive valve fibrosis, calcification,

and stenosis. Multi-omics approaches, including transcriptomics and proteomics, have been successfully applied to select human AV tissue to identify novel putative regulatory networks for calcific aortic valve disease.(24-28) However, few studies have interrogated the proteomic correlates of AV hemodynamics and progression in large human cohorts. Such data may provide novel insights into mechanisms underlying AS and facilitate early prediction of persons at risk for the development of significant AS.(29) We applied serial high-throughput plasma proteomics in large community based cohort with follow-up for incident AV events and detailed AV hemodynamic phenotyping in late-life.

Methods

Study Population

The Atherosclerosis Risk in Communities (ARIC) study is a community-based prospective epidemiologic cohort study, the design and methods of which have been previously described.(30) Between 1987 and 1989, 15,792 middle-aged subjects were enrolled in 4 communities in the United States: Forsyth County, NC, Jackson, MS, suburban Minneapolis, MN, and Washington County, MD. Participants underwent four study visits between 1987 and 1998, and subsequently returned for the fifth (2011-2013), sixth (2016-2017), and seventh (2018-2019) study visits. The ARIC study protocol was approved by institutional review boards at all 4 field centers. All participants provided written informed consent. In this analysis, we studied: (a) 11, 430 participants attended study Visit 3 (1993-1995), were free of prior hospitalization with an AV disease or intervention ICD code, and had available proteomic data; and (b) 4,899 participants who attended study Visit 5 (2011 to 2013), were free of prior AV replacement, underwent echocardiography with adequate images for assessing the peak velocity at AV, and had available proteomic data. **(Figure 1; Supplemental Figure 1).**

Plasma Proteomics

The plasma proteins of ARIC participants' blood samples taken at visit 3 and visit 5 had been stored frozen at −80 °C and measured by the SOMAscan version 4 assay developed by SomaLogic. DNA microarray technology was used in this platform to quantify 5,248 single-strand DNA-based aptamers that bind to targeted proteins. After excluding the quality control outliers, 4,877 aptamers measuring 4,697 unique proteins or protein complexes were analyzed. For some proteins, duplicated aptamers could be present [e.g., Sushi von Willebrand factor type A, EGF, and pentraxin domain-containing protein 1 (SVEP1)]. Relative fluorescence units were used to quantify the proteins and the values were normalized through a pool of healthy control participants and standardized. (31-34) The reproducibility and quality control of the proteins measured in ARIC were described before. (31, 32)

Echocardiography Measures of AS severity

Procedures for echocardiography in ARIC at Visit 5, including reproducibility metrics, have been previously described, and were equivalent at Visit 7.(35) Studies were acquired by certified sonographers using uniform imaging machines (Philips iE33, Koninklijke Philips, The Netherlands), and probes (Philips XMatrix). All quantitative measures were over-read by staff cardiologists at the Brigham and Women's Hospital. Aortic valve peak velocity (Vmax) and velocity-time integral (VTI) were measured using continuous-wave (CW) Doppler from the apical 5 chamber view, and left ventricular outflow tract (LVOT VTI) was measured using pulse wave Doppler. The dimensionless index (DI) was calculated as the ratio between LVOT VTI and AV VTI. (36, 37) LVOT diameter was measured in the parasternal long-axis view. LVOT cross-

sectional area (CSA_{LVOT}) was calculated as 3.14*(LVOT diameter/2). The AVA was calculated using the continuity equation as follows: AVA = CSA_{LVOT}* VTI_{LVOT} / VTI_{AV}.(35, 37). Stroke volume (SV) was calculated as CSA_{LVOT}* VTI_{LVOT}. Both AVA and SV were indexed using the calculated body surface area.

Incident Aortic Valve Events

ARIC cohort participants undergo active surveillance for hospitalizations with abstraction of all hospitalization International Classification of Disease (ICD) discharge codes. AV ICD codes used to identify AV disease and AV interventions. AV disease included ICD-9 code 424.1 (38) and ICD-10 codes 135.1, I35.0, and I35.2 (38, 39) that have been previously validated. (5, 40) AV interventions ICD codes included operative interventions and Transcatheter Aortic Valve Implantation (TAVI) and they also were validated before (**Supplementary material**). Codes for non-specific rheumatic fever-related diseases were not included.

Hospitalizations with an AV related ICD discharge code occurring prior to the Visit 3 date were used to exclude participants for Visit 3 analyses. Incident AV events post-Visit 3 were assessed through December 31, 2019.

Late-life AV Calcification

Non-contrast cardiac-gated computed tomography (CT) was systematically conducted in a sub-set of ARIC at the time of Visit 7 who were free from coronary heart disease in 2016. Among Visit 5 participants with included in this analysis, 1,802 had quantitative data on AV calcification by CT at the time of Visit 7. The acquisition was performed using Siemens Somatom Cardiac 64 devices in three study centers, and a GE 64-slice PET/CT device in one center. The evaluation of

calcification was based on the Agatston score of AV leaflets, with calcification defined as lesions with attenuation ≥130 Hounsfield Units (HU) and area ≥1 mm2 in each slice level as previously described.(41)

Clinical Covariates

Coronary heart disease prevalence at Visit 3 and Visit 5 was based on ARIC surveillance, abstraction, and adjudication of hospitalizations with coronary heart disease-related ICD codes as previously described.(30, 42) Prevalent atrial fibrillation events was ascertained based on atrial fibrillation-related ICD codes and visit electrocardiograms.(43) Heart failure was ascertained based on heart failure-associated ICD hospitalization codes up to 2005, and employed additional chart abstraction and physician adjudication as previously described. (44, 45) Prevalent hypertension was ascertained based on participant report of blood pressure medication use or blood pressure ≥140/90 mm Hg at study Visit. Prevalent diabetes mellitus was ascertained based on self-report of a physician's diagnosis of diabetes mellitus, antidiabetic medication use, fasting glucose ≥126 mg/dL, or non-fasting glucose ≥200 mg/dL at study visits. Body mass index was calculated from weight and height assessed at each visit.(46)

Statistical analysis

Among Visit 5 participants (late-life sample), we identified proteins associated cross-sectionally with Vmax at a false discovery rate (FDR) adjusted p-value <0.05 using multivariable linear regression. Models adjusted for age, sex, race, Visit 5 heart rate, systolic blood pressure, hypertension, diabetes, coronary heart disease, heart failure, atrial fibrillation, and body mass index. We employed Multiple Imputation by Chained Equation method to account for missing

covariate values with a frequency of <5%. For the resulting 917 candidate proteins, we used proteomic measures at Visit 3 (mid-life sample) to test their association with incident AV events using multivariable Cox proportional hazard models at an FDR-adjusted p value <0.05. Model covariates included age, sex, race, Visit 3 heart rate, systolic blood pressure, hypertension, diabetes, coronary heart disease, heart failure, atrial fibrillation, and body mass index. This resulted in 72 candidate proteins cross-sectionally associated with peak AV velocity in late-life (Visit 5) and with incident AV events in mid-life (Visit 3).

As peak velocity is dependent on stroke volume, we then identified the subset of these proteins that also associated with cross-sectionally with the DI at Visit 5 at an FDR-adjusted p value <0.05 in multivariable linear regression models. We then assessed the associations of these proteins with AV calcification by CT at Visit 7 and with change in Vmax between Visits 5 and 7. Associations with AV calcification were assessed using multivariable ordinal regression, with calcification extent categorized into four groups based on Agatston score due to its right skewed distribution: 0 value and tertiles of non-zero values (tertile 1 34.12, tertile 2 134.38, tertile 3 3816.80). For associations with change in peak AV velocity, we employed multivariable linear regression models adjusting for Models adjusted for age, sex, race, Visit 5 heart rate, systolic blood pressure, hypertension, diabetes, coronary heart disease, heart failure, atrial fibrillation, body mass index, in addition to Visit 7 heart rate and systolic blood pressure.

To explore potential biological mechanisms relating candidate proteins to AS, we employed the Ingenuity Pathway Analysis (IPA; QIAGEN Inc) Core Analysis package (47) to identify enriched canonical pathways, protein networks, and upstream regulators from the key

candidate proteins associated with AV peak velocity, DI, and incident AV events. A hypothesis generating *de novo* pathway was created by combing the Upstream Regulator Analysis with the available downstream Calcific Aortic Stenosis disease network to visualize how the proposed upstream regulators could be related to AS.(48)

All analyses were performed using R statistical software (V 4.1.2), and STATA 16. P-values after correction using a false discovery rate (FDR) of less than 0.05 were considered significant. FDR method described by Benjamini and Hochberg.(38)

Results

Among the 4,899 participants included at Visit 5, mean age was 76 ± 5 years, 43% were male, 18% were Black participants. **(Table1)**. The mean peak AV velocity was1.3 m/sec (range 0.64 -5.9 m/sec). Higher peak AV velocity was associated with higher prevalence of cardiovascular risk factors and prevalent CV diseases (**Supplemental Table1**). In multivariable linear regression models, 917 of 4,877 proteins at Visit 5 were cross-sectionally associated with peak AV velocity at FDR <0.05 **(Figure 2A; Supplemental Table 2)**.

Among the 11,430 participants included at Visit 3, mean age 60 ± 6 years, 46% were male, and 21% were Black participants **(Table1)**. Over a median follow-up. 22.2 years (IQR: 14.4 – 24.8), 912 participants were hospitalized with ICD codes related to AS diagnosis or intervention. Compared to those that did not experience an AS hospitalization, participants with an AS hospitalization during follow-up were older, more frequent men and of White race, and had higher prevalence of cardiovascular risk factors and cardiovascular diseases at Visit 3 **(Supplemental Table 3)**. In multivariable Cox proportional hazards regression models, 72 of the 917 proteins cross-sectionally associated with Vmax at Visit 5 were also associated with post-Visit 3 incident AV hospitalizations at FDR <0.05 **(Figure 2B**; **Supplemental Table 4**). For all

but one candidate protein (Signal Recognition Particle 14), directionally consistent associations were observed such that proteins associated with higher Vmax were also associated with higher risk of an incident AV event, and vice versa (**Figure 2C**)

Of these 72 proteins, 52 proteins were associated with DI **(Figure 2D, Supplemental Table 5)**. Based on their values at Visit 5, these 52 proteins comprised three clusters based on component measures of aortic stenosis severity (**Figure 3A**). The 14 proteins in cluster 1 were associated with lower AV gradient, higher DI, and higher AVA. Proteins in the remaining two clusters were associated with higher AV gradient, lower DI, and lower AVA, with the 9 proteins in Cluster 2 demonstrating the most robust associations, including Macrophage metalloelastase (MMP-12). Of these 52 proteins, assessed at Visit 5, 38 were associated with AV calcification by CT at Visit 7 in ordinal regression models at a FDR adjusted $p<0.05$ with the highest odds observed for MMP12 (adjusted OR 1.24 [95% CI 1.18-1.31], $p=1.1*10^{-16}$) and Growth/differentiation factor 15 (GDF-15) (1.21 [1.15-1.28], $p=1.3*10^{-12}$; **Figure 3B, Supplemental Table 6**). Visit 5 values of two of the 52 proteins were significantly associated with an increase in Vmax over 6.3 ± 0.6 years between Visits 5 and Visit 7 (n=2,314): MMP-12 (adjusted beta coefficient 2.64 [95% CI 1.08, 4.20], p=0.00092) and Secreted and transmembrane protein 1 (SECTM1; 2.52 [1.00, 4.04], p=0.0012; **Figure 3C, Supplemental Table 7**).

In pathway analysis, the 52 candidate proteins enriched for several Canonical Pathways, including liver X receptors (LXRs) / retinoid X receptors (RXRs) Activation pathway **(Figure 4A)**, and for five different networks associated with Connective Tissue Development and Function, Cellular Growth and Proliferation, Lipid Metabolism, Immune Cell Trafficking, and Inflammatory Disease **(Supplemental Table 8).** Upstream analysis suggested Interferon Gamma (IFNG) as an upstream regulator **(Figure 4B)**, with nine of the ten related proteins significantly

associated with AV calcium at Visit 7. In a complementary analysis, we identified 22 molecules associated with calcific AS based on IPA databases **(Supplemental Figure 2)**, 11 of which had direct and indirect relationships with 32 of our candidate proteins **(Figure 5A)**. Based on the previous Upstream and complementary analyses, a hypothesized Regulator Effects diagram was generated to explain how calcific AS is regulated in the data set by the activated upstream regulator. Potentially related drugs were added to the diagram **(Figure 5B)**

Discussion

Using high-throughput proteomics, detailed cardiac phenotyping, and prospective ascertainment of AV hospitalizations, we identified 52 circulating proteins significantly associated with both AV hemodynamics and AS severity by echocardiography (Vmax and DI) when assessed cross-sectionally in late-life (Visit 5) and with incident AS related hospitalization when ascertained in mid-life (Visit 3). Of these, 14 proteins were associated with less severe AS, and among the remainder, a subset of 9 proteins demonstrated particularly robust associations with greater AS severity. Of the 52 proteins, 38 also demonstrated consistent associations with magnitude of AV calcification by CT 6 years later and 2 were associated with changes in Vmax over 6 years. MMP-12 emerged as a particularly robust and consistent marker of AS severity, with higher MMP-12 levels demonstrating robust associations with incident AV hospitalizations when assessed in mid-life, worse AV hemodynamics cross-sectionally (higher Vmax, lower DI, lower AVA) in late-life, greater increase in Vmax over 6 years in late-life, and greater degree of AV calcification in late-life. Pathway analysis suggested interferon-gamma as a potential upstream regulator. Together, these findings highlight a potential novel biomarker for AS risk, and a novel putative targetable pathway to prevent AS progression.

Previous transcriptomic and proteomic studies of diseased AV tissue extracted during valve replacement have provided important insights into the cellular and molecular mechanisms responsible for calcific AV stenosis. (24, 27, 49-52) For example, by performing transcriptomic and proteomic characterization of distinct pathologic stages represented in excised calcific AV following valve replacement, Schlotter et al identified partially distinct proteomic profiles between the non-diseased, fibrotic, and calcific layers, with the later two enriched for inflammation and calcification-related pathways.(24)

Existing studies of circulating proteins and calcific aortic valve disease have similarly focused on patients with advanced – typically severe – aortic stenosis. Several early studies were too small to draw meaningful conclusions (n <10) (53, 54). In a study comparing 60 patients with severe calcific aortic stenosis undergoing valve replacement to 20 healthy controls, serum and valve tissue concentrations of targeted proteins associated with inflammation, collagen turnover, and calcification were associated with AS.(55) In contrast, in a catheterization laboratory based study of 80 patients with severe AS compared to 1,164 referred patients without severe AS, from a 109 protein panel only N-terminal pro-B-type natriuretic peptide, von Willebrand factor and fetuin-A were identified that optimized discrimination of patients with versus without severe AS.(56) Perhaps most similar to our study, in a recent nested case-control study of the Northern Sweden Health and Disease Stuy that included 334 participants with incident AV replacement match 1:2 with controls, five out of 92 assessed proteins at baseline were associated with incident AS case status, including GDF-15. (57) However, coronary artery disease may have confounded these associations, as associations did not replicate in sensitivity analyses restricted to participants free of coronary disease. Our study extends substantially on these prior analyses by leveraging a large cohort of community-based participants, assessing

more than 4,800 proteins, and interrogating associations with aortic valve hemodynamics by echocardiography, AV calcification by CT, and incident clinical AV events in persons without significant AS at baseline.

Cumulative evidence indicate that matrix metalloproteinases (MMPs), which are enzymes produced by inflammatory cells to degrade collagen, elastin, and proteoglycans are associated with AS progression.(58-62) MMP9 and proMMP2 are over-expressed in the aortic tissue valve (59, 60), whereby the serum level of MMP1 and MMP10 were discovered to be associated with AS progression(58, 63). A previous transcriptomic study showed that MMP12 is the highest gene upregulated in calcific AS(64), but this genetic association was not found in rheumatic AS.(65) In an experimental model of aortic Valve Interstitial Cells (VIC) cell culture, MMP-12 induced pro-osteogenic responses and increased the level of their markers like ALP. Up to our knowledge, no study has defined MMP12 as circulatory biomarkers, and non-specific drugs like doxycycline failed to decrease its level in the animal model.(66) Marimastat and Ilomastat are synthetic broad-spectrum MMPs inhibitors used in humans for cancer treatment and have an anti-inflammatory effect in cystic fibrosis mice.(67) A more specific molecules for MMPs inhibition with different doses and time frames were suggested by Matilla et.al.(63)

Interestingly, GDF15 showed the highest HR for post Visit 3 (mid-life sample) AS related events and was significantly associated with AV calcification in late life. The evidence of the prognostic importance of GDF15 is increasing (68-72), however, it is involved in inflammation and apoptotic pathways, which are key processes in fibrosis development and proved to be a predictor of coronary artery disease progression (13, 73) GDF15 is family member of factor-β (TGF-β) superfamily which was reported to drive a cascade of events

involved in calcific AS pathogenesis, including apoptosis and formation of pre-calcific alkaline phosphatase enriched nodules. (20, 74, 75)

Pathway analysis incorporating key candidate proteins identified in our analysis identified IFNG as a key upstream regulator, which demonstrated significant interactions with nine candidate proteins including MMP-12 and GDF-15 (**Figure 5B**). $CD8^+$ T cells which secret IFNG commonly infiltrate the calcific AV, and $CD14^+$ monocytes stimulation by IFNG drive the osteoclast-like cell activity toward more calcification of cardiac tissue. (9, 76, 77) In VIC culture from AV tissue of cardiac transplant recipients, Parra-Izquierdo et al. demonstrated the pathological role of IFNG in calcific AS and suggested that available Janus kinase (JAK) inhibitors as potential safe therapeutic solution for AS progression by inhibiting JAK/STAT pathways.(78) Notably, one of our candidate proteins also associated with change in peak AV velocity over 6 years in late-life, SECTM1, appears to be induced by IFNG indirectly through Signal transducer and activator of transcription 1 (STAT1).(79)

This study has several limitations. Despite the large sample size and consistency of associations across multiple timepoints and AS-related end-points (echocardiographic, CT-based, clinical events), Lipoprotein(a) that had known association with AS was not available among the scanned proteins and the generalizability of results is limited by lack of external validation in an independent cohort different from ARIC. Despite multivariable adjustment for all analyses, we are unable to assess the causal relationship of the proteomic associations due to the risk of residual confounding. As we employed an aptamer affinity discover platform for proteomic measurements, absolute quantification of protein concentration is not available and all analyses were performed using relative fluorescence unit values. AV hospitalization events were based on ICD codes and were not adjudicated. For associations with CT-based AV calcification at Visit 7

and change in Vmax from Visit 5 to Visit 7, non-random Visit 7 non-attendance among Visit 5 participants may bias our results toward the null. In addition, the sampling strategy for Visit 7 CT, which focused on participants free of prevalent coronary artery disease, likely contributed to the high proportion of participants with a zero calcium score, limiting the power of its analysis. Finally, the pathway analyses, including the Upstream Regulator analysis, are hypothesis-generating and require further validation.

Conclusions

Using serial high throughput proteomics, multimodality image, and active event surveillance in a thoroughly phenotyped longitudinal cohort study, we identified 38 circulating proteins with robust associations with AV hemodynamics, calcification, and risk of incident AV-related hospitalizations. Higher MMP12 values demonstrated particularly robust and consistent associations with worse AV hemodynamics, progression in AV over time, magnitude of AV calcification, and risk of incident AV events. Pathway analysis identified IFNG as a potentially important upstream regulator related to 9 of 38 identified proteins, including MMP12. These findings highlight a potential novel biomarker for AS risk, and a novel putative targetable pathway to prevent AS progression.

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Table 1: Characteristics of ARIC participants at Visit 3 and Visit 5

	Visit 3 free from aortic valve events	**Visit 5 with echo**
n	**11430**	**4899**
Age (mean (SD))	60 (6)	76 (5)
Black (%)	2435 (21)	875 (18)
Male (%)	5199 (46)	2115 (43)
DM (%)	1811 (16)	1826 (37)
HTN(%)	4691 (41)	4036 (82)
HF (%)	568 (5)	231 (5)
CHD (%)	833 (7)	935 (20)
AF (%)	129 (1)	344 (7)
BMI (mean (SD))	29 (6)	29 (6)
HR (mean (SD))	66 (10)	62 (10)
SBP (mean (SD))	125 (19)	130 (18)

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protein 2 (RBL2), Monocyte differentiation antigen CD14 (CD14), Insulin-like growth factor-binding protein 2 (IGFBP2), Afamin (AFM), Collagen alpha-1(XVIII) chain (COL18A1), Complement component C9 (C9), Triggering receptor expressed on myeloid cells 2 (TREM2), Calsyntenin-3 (CLSTN3), Protein SET (SET), Beta-2-microglobulin (B2M), Tumor necrosis factor receptor superfamily member 1A (TNFRSF1A), Tumor necrosis factor receptor superfamily member 1B (TNFRSF1B), Gamma-aminobutyric acid receptor-associated protein (GABARAP), Neurocan core protein (NCAN), Leptin (LEP), Angiopoietin-2 (ANGPT2), Epidermal growth factor receptor (EGFR), Tryptophan--tRNA ligase, cytoplasmic (WARS), Macrophage metalloelastase (MMP12), RNA-binding protein EWS (EWSR1), Alpha-2-HS-glycoprotein (AHSG), Growth hormone receptor (GHR), WAP four-disulfide core domain protein 2 (WFDC2), Thrombospondin-2 (THBS2), Thioredoxin domain-containing protein 5 (TXNDC5), MAM domain-containing glycosylphosphatidylinositol anchor protein 2 (MDGA2), Cystatin-C (CST3), Complement C1q tumor necrosis factor-related protein 1 (C1QTNF1), Trefoil factor 2 (TFF2), Lithostathine-1-alpha (REG1A), Natriuretic peptides B (NPPB), Growth/differentiation factor 15 (GDF15).

Figure 1. Study design

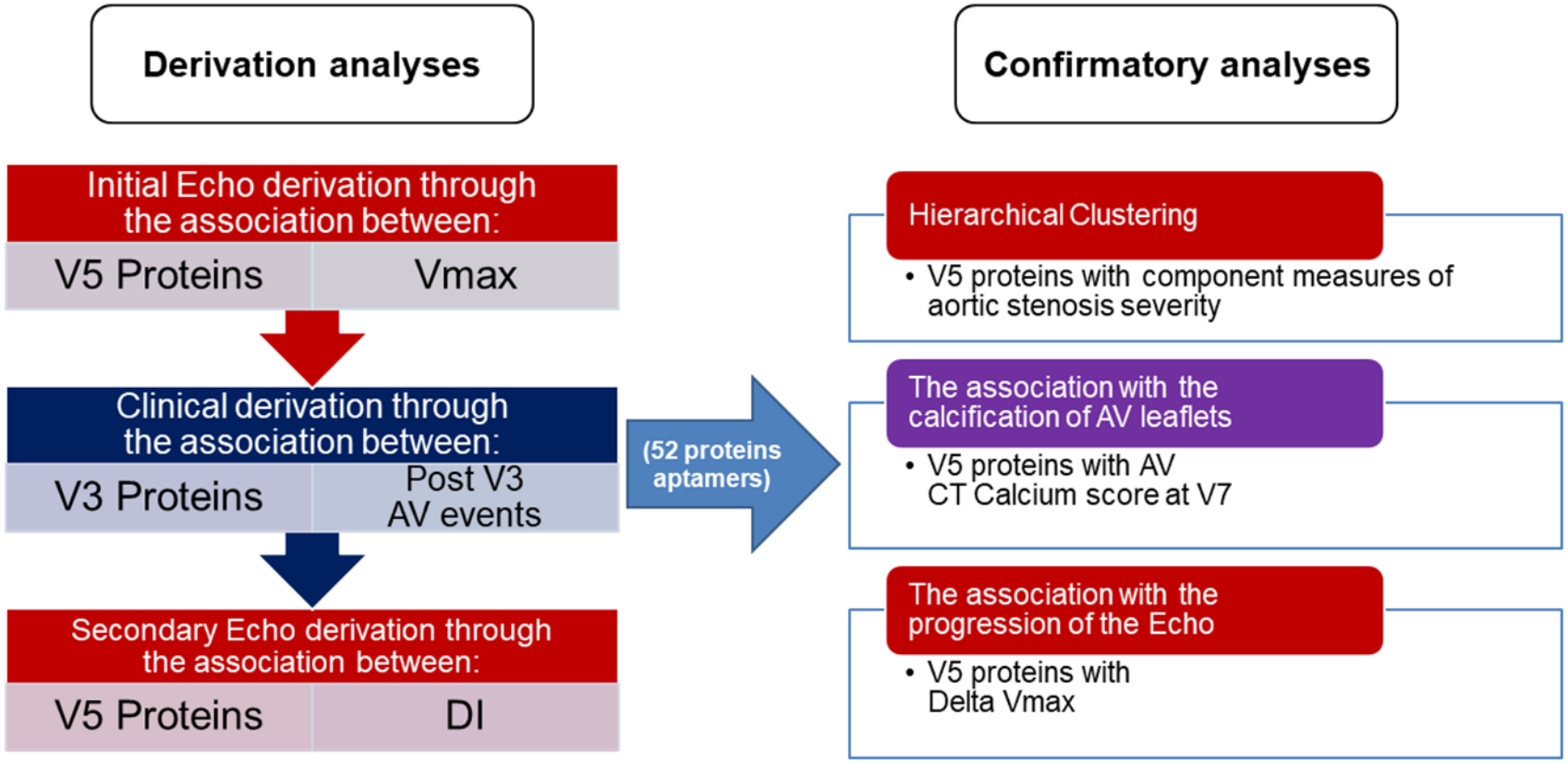
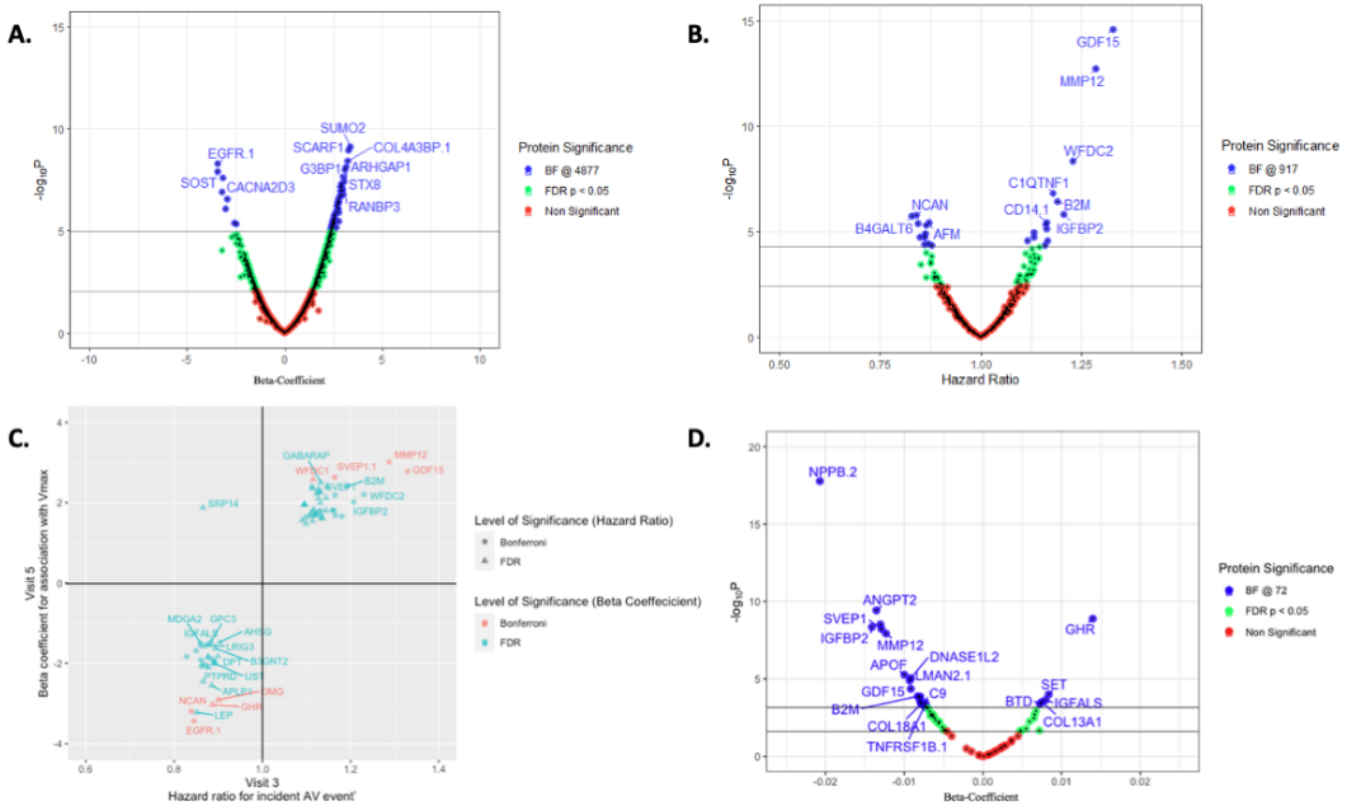
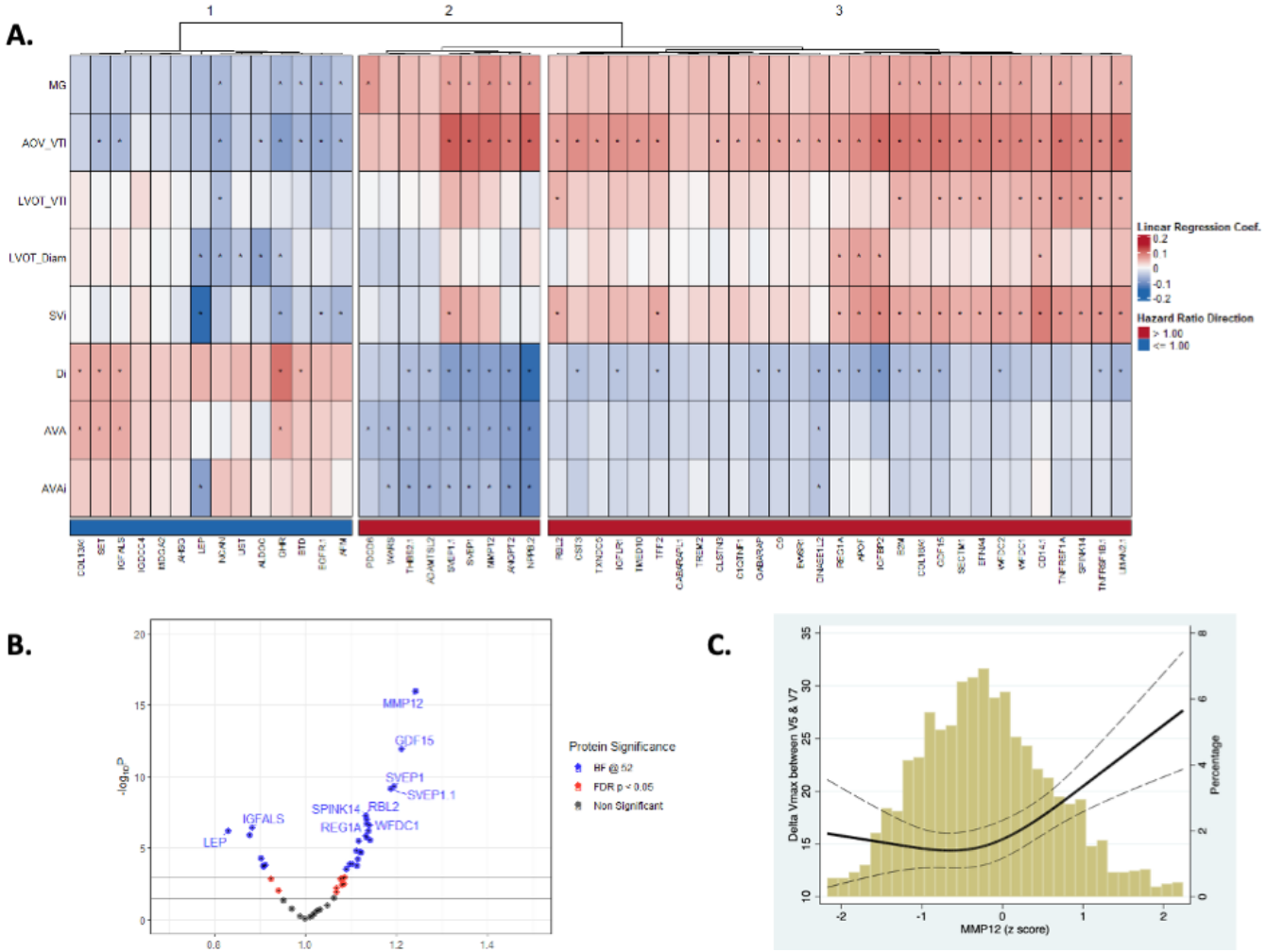


Figure 2. Associations of proteomic measures with AV hemodynamics and risk of incident AV-associated hospitalization. Panel A: Volcano plot of cross-sectional associations with Visit 5 aortic valve peak velocity; Panel B: Volcano plot of associations with post-Visit 3 Aortic valve events; Panel C: Plot of beta-coefficient for association with AV peak velocity at Visit 5 from multivariable linear regression models (X-axis) and hazard ratio for incident AV-related hospitalization post-Visit 3 from multivariable Cox proportional hazard regression models (Y-axis); Panel D: Volcano plot of cross-sectional associations with Visit 5 dimensionless index among 72 protiens associated with both peak AV velocity at Visit 5 and incident AV-related hospitalization post-Visit 3.



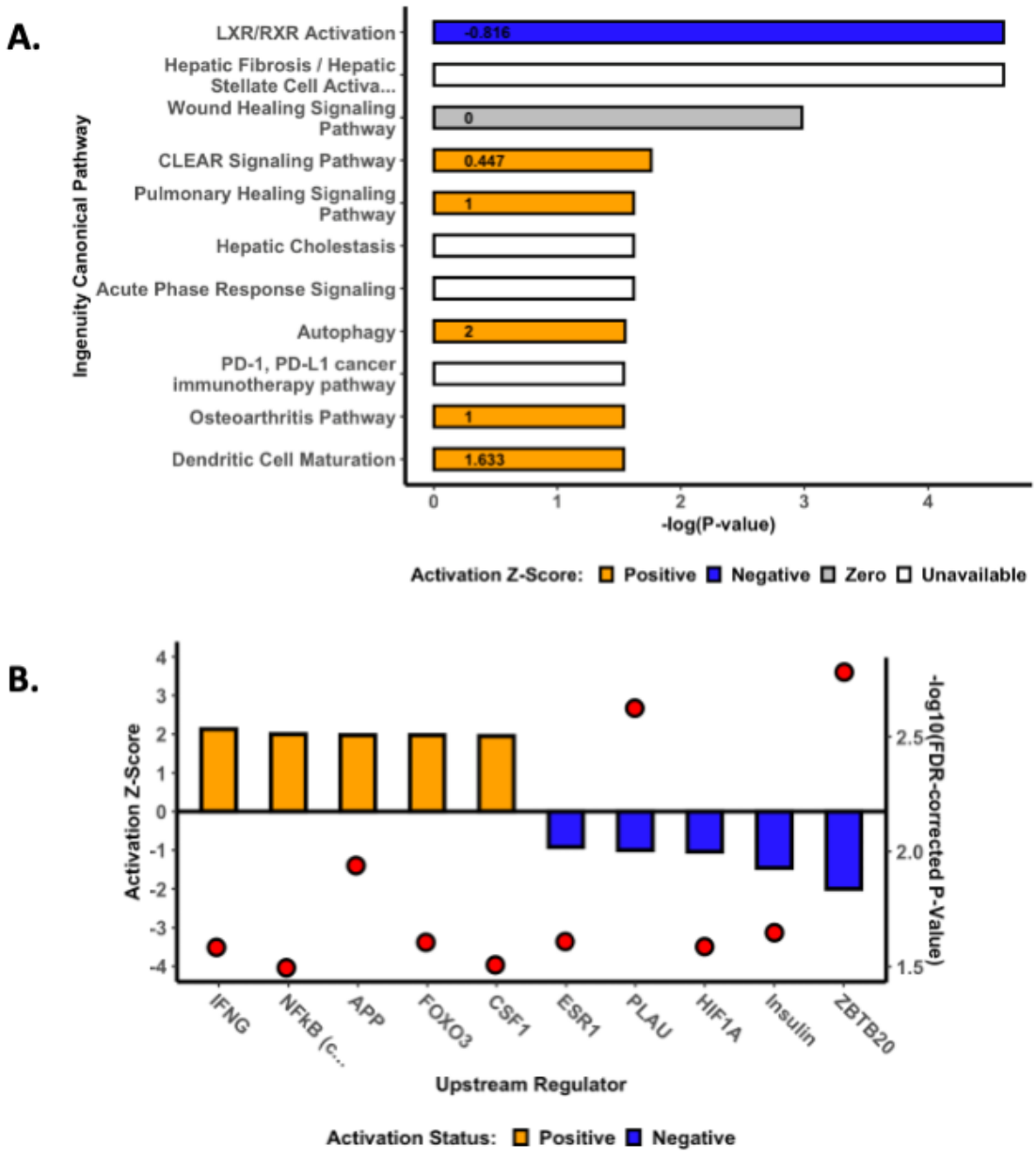
Linear regression models adjusted for Visit 5 age, sex, race, hypertension, diabetes, heart failure, coronary heart disease, atrial fibrillation, systolic blood pressure, heart rate, body mass index; Cox regression models adjusted for Visit 3 age, sex, race, hypertension, diabetes, heart failure, coronary heart disease, atrial fibrillation, systolic blood pressure, heart rate, body mass index

Figure 3. Associations of candidate AS-associated proteins with echocardiographic AV parameters, AV calcification, and longitudinal change in peak aortic valve velocity. Panel A: Heatmap of echocardiographic metrics of AV hemodynamics at Visit 5 for 52 key candidate proteins; Panel B: Volcano plot of associations with aortic valve calcification at Visit 7 using multivariable ordinal regression; Panel C: Restricted cubic spline of association of Visit 5 MMP12 level with the change in Vmax from Visit 5 to Visit 7.



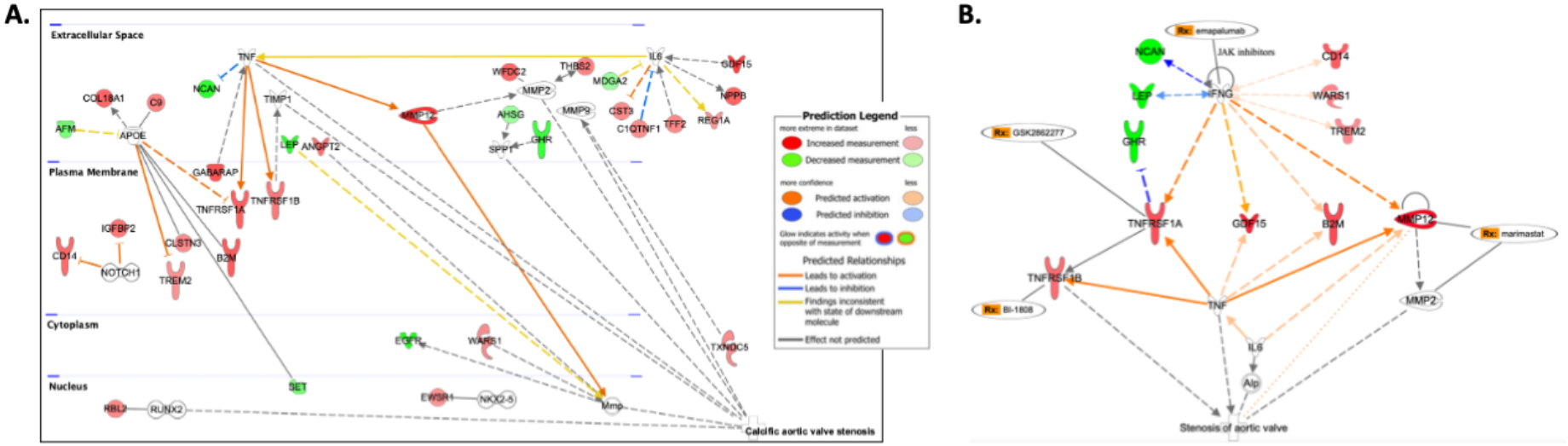
Linear regression models adjusted for Visit 5 age, sex, race, hypertension, diabetes, heart failure, coronary heart disease, atrial fibrillation, systolic blood pressure, heart rate, body mass index

Figure 4. Pathway enrichment and upstream regulator analysis. Panel A: Results of the Ingenuity Canonical Pathway analysis based on the 52 proteins candidates. Panel B: Results of the Ingenuity Upstream Regulator Analysis.



Gray bar – no prediction can be made (pathway currently ineligible for a prediction). LXRs/ RXRs (Liver X receptors / retinoid X receptors), APP (amyloid precursor protein), CSF1 (Colony Stimulating Factor 1), ESR1 (Estrogen Receptor 1), FOXO3 (factor forkhead box O-3), HIF1A (Hypoxia Inducible Factor 1 Subunit Alpha), IFNG (Interferon Gamma), PLAU (Plasminogen Activator, Urokinase), ZBTB20 (Zinc Finger And BTB Domain Containing 20)

Figure 5: Potential pathways linking candidate proteins to calcific AS. Panel A: The simplified relationship between the Calcific Aortic Valve Stenosis Disease Ingenuity Network with the 52 candidate protein subset. The uncolored molecules represent the molecules that were not included in the 52 candidate protein. Panel B: Simplified hypothesis-generating diagram for the Ingenuity Regulator Effects Analysis



In Panel B, the Rx label indicates the possible drugs that can affect each molecule in the network. The uncolored molecules are not present in the subset of 52 candidate proteins. We proposed a new relationship between MMP12 and AS (small dotted line). Retinoblastoma-like protein 2 (RBL2), Monocyte differentiation antigen CD14 (CD14), Insulin-like growth factor-binding protein 2 (IGFBP2), Afamin (AFM), Collagen alpha-1(XVIII) chain (COL18A1), Complement component C9 (C9), Triggering receptor expressed on myeloid cells 2 (TREM2), Calsyntenin-3 (CLSTN3), Protein SET (SET), Beta-2-microglobulin (B2M), Tumor necrosis factor receptor superfamily member 1A (TNFRSF1A), Tumor necrosis factor receptor superfamily member 1B (TNFRSF1B), Gamma-aminobutyric acid receptor-associated protein (GABARAP), Neurocan core protein (NCAN), Leptin (LEP), Angiopoietin-2 (ANGPT2), Epidermal growth factor receptor (EGFR), Tryptophan--tRNA ligase, cytoplasmic (WARS), Macrophage metalloelastase (MMP12), RNA-binding protein EWS (EWSR1), Alpha-2-HS-glycoprotein (AHSG), Growth hormone receptor (GHR), WAP four-disulfide core domain protein 2 (WFDC2), Thrombospondin-2 (THBS2), Thioredoxin domain-containing protein 5 (TXNDC5), MAM domain-containing glycosylphosphatidylinositol anchor protein 2 (MDGA2), Cystatin-C (CST3), Complement C1q tumor necrosis factor-related protein 1 (C1QTNF1), Trefoil factor 2 (TFF2), Lithostathine-1-alpha (REG1A), Natriuretic peptides B (NPPB), Growth/differentiation factor 15 (GDF15).

Supplemental Materials

Supplement

ICD codes of aortic valve (AV)related intervention:

the procedures ICD codes included : (ICD-9-PCS codes) 35.11(1), 35.21, 35.22 (2); or (ICD-10-PCS codes) 02NFxx, 027Fxx, 02QFxx, 02RFxx, , 02RF0xx, 02RF4xx, X2RF0xx, 02NGxx, 027Gxx, 02QGxx, 02VGxx, 02RGxx, 027Gxx (1), 02RF37H, 02RF38H, 02RF3J, 02RF3KH, 02RF37Z, 02RF38Z, 02RF3JZ or 02RF3KZ. (3)

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- ***Supplemental Table 1:*** *Visit 5 characteristics by quartile of peak velocity*

Characteristics at Visit 5	1st Vmax Quartile	2nd Vmax Quartile	3rd Vmax Quartile	4th Vmax Quartile	
n	1225	1225	1225	1224	P-Value
Age (mean (SD))	75 (5)	75 (5)	75 (5)	76 (5)	<0.001
Black (%)	251 (21)	228 (19)	220 (18)	176 (14)	0.001
Male (%)	557 (46)	509 (42)	500 (41)	549 (45)	0.043
DM (%)	398 (33)	437 (36)	476 (39)	515 (42)	<0.001
HTN (%)	966 (79)	993 (8)	1000 (82)	1077 (88)	<0.001
HF (%)	55 (5)	36 (3)	49 (4)	91 (7)	<0.001
CHD (%)	219 (19)	210 (18)	219 (19)	287 (25)	<0.001
AF (%)	95 (8)	55 (5)	72 (6)	122 (10)	<0.001
BMI (mean (SD))	27 (5)	28 (5)	29 (6)	30 (6)	<0.001
HR (mean (SD))	64 (10)	61 (10)	62 (10)	62 (11)	<0.001
SBP (mean (SD))	128 (18)	130 (18)	131 (18)	131 (18)	0.001

- ***Supplemental Table 2****: Individual protein aptamers name, beta coefficient, CI, p-value from linear models for associated with Vmax*

name	gene	Model 2	Model1
Epidermal growth factor receptor	EGFR.1	-3.438 (-4.589, -2.286) p=5.20e-09	-5.252 (-6.367, -4.137) p=3.74e-20
Neurocan core protein	NCAN	-3.190 (-4.373, -2.008) p=1.29e-07	-5.375 (-6.482, -4.267) p=2.78e-21
Macrophage metalloelastase	MMP12	3.013 (1.884, 4.143) p=1.75e-07	3.362 (2.239, 4.484) p=4.64e-09
Growth hormone receptor	GHR	-3.031 (-4.234, -1.827) p=8.25e-07	-1.076 (-2.226, 0.073) p=6.64e-02
Growth/differentiation factor 15	GDF15	2.778 (1.605, 3.951) p=3.53e-06	3.874 (2.744, 5.005) p=2.06e-11
WAP four-disulfide core domain protein 1	WFDC1	2.579 (1.481, 3.677) p=4.22e-06	3.298 (2.205, 4.390) p=3.47e-09
Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	SVEP1.1	2.632 (1.482, 3.782) p=7.39e-06	3.503 (2.377, 4.630) p=1.16e-09
Gamma-aminobutyric acid receptor-associated protein	GABARAP	2.499 (1.372, 3.626) p=1.41e-05	4.371 (3.283, 5.459) p=4.10e-15
Ephrin-A4	EFNA4	2.390 (1.299, 3.481) p=1.78e-05	3.650 (2.571, 4.729) p=3.74e-11
Programmed cell death protein 6	PDCD6	2.230 (1.181, 3.280) p=3.16e-05	1.706 (0.641, 2.770) p=1.69e-03
Beta-2-microglobulin	B2M	2.396 (1.262, 3.529) p=3.49e-05	3.890 (2.784, 4.996) p=6.08e-12
Vesicular integral-membrane protein VIP36	LMAN2.1	2.340 (1.224, 3.456) p=4.01e-05	3.301 (2.193, 4.410) p=5.58e-09
Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	SVEP1	2.392 (1.233, 3.551) p=5.29e-05	3.195 (2.059, 4.330) p=3.66e-08
Tumor necrosis factor receptor superfamily member 1A	TNFRSF1A	2.354 (1.205, 3.503) p=6.03e-05	4.199 (3.084, 5.314) p=1.81e-13
Leptin	LEP	-3.221 (-4.837, -1.605) p=9.47e-05	2.801 (1.562, 4.039) p=9.49e-06
Collagen alpha-1(XVIII) chain	COL18A1	2.254 (1.109, 3.400) p=1.16e-04	2.963 (1.826, 4.100) p=3.36e-07
Monocyte differentiation antigen CD14	CD14.1	2.188 (1.066, 3.309) p=1.33e-04	1.756 (0.631, 2.881) p=2.23e-03
WAP four-disulfide core domain protein 2	WFDC2	2.203 (1.045, 3.361) p=1.93e-04	2.501 (1.348, 3.655) p=2.16e-05
Biotinidase	BTD	-2.014 (-3.080, -0.948) p=2.15e-04	-2.051 (-3.129, -0.972) p=1.95e-04
Angiopoietin-2	ANGPT2	2.117 (0.985, 3.248) p=2.47e-04	3.889 (2.808, 4.969) p=1.99e-12
Afamin	AFM	-2.051 (-3.154, -0.949) p=2.66e-04	-1.234 (-2.332, -0.135) p=2.78e-02
Protein SET	SET	-2.075 (-3.195, -0.955) p=2.84e-04	-3.328 (-4.441, -2.214) p=4.93e-09
Uronyl 2-sulfotransferase	UST	-1.994 (-3.076, -0.911) p=3.09e-04	-3.004 (-4.087, -1.922) p=5.50e-08
Serine protease inhibitor Kazal-type 14	SPINK14	1.951 (0.891, 3.010) p=3.11e-04	2.716 (1.647, 3.784) p=6.43e-07
Natriuretic peptides B	NPPB.2	2.274 (1.035, 3.513) p=3.24e-04	3.112 (1.952, 4.271) p=1.49e-07
Deoxyribonuclease-1-like 2	DNASE1L2	1.958 (0.877, 3.039) p=3.89e-04	3.106 (2.026, 4.186) p=1.81e-08
Tumor necrosis factor receptor superfamily member 1B	TNFRSF1B.1	1.996 (0.884, 3.109) p=4.36e-04	3.504 (2.409, 4.598) p=3.80e-10
Immunoglobulin superfamily DCC subclass member 4	IGDCC4	-1.913 (-3.003, -0.824) p=5.78e-04	-3.250 (-4.332, -2.168) p=4.20e-09
Complement component C9	C9	1.800 (0.729, 2.872) p=9.95e-04	1.974 (0.892, 3.056) p=3.53e-04
Secreted and transmembrane protein 1	SECTM1	1.802 (0.721, 2.883) p=1.09e-03	2.547 (1.467, 3.628) p=3.90e-06

ADAMTS-like protein 2	ADAMTSL2	1.809 (0.690, 2.927) p=1.54e-03	3.692 (2.620, 4.764) p=1.61e-11
IGF-like family receptor 1	IGFLR1	1.761 (0.671, 2.851) p=1.55e-03	3.132 (2.054, 4.211) p=1.32e-08
Insulin-like growth factor-binding protein 2	IGFBP2	2.017 (0.758, 3.276) p=1.70e-03	-0.335 (-1.522, 0.852) p=5.80e-01
Thrombospondin-2	THBS2.1	1.812 (0.673, 2.951) p=1.82e-03	3.730 (2.639, 4.822) p=2.31e-11
Retinoblastoma-like protein 2	RBL2	1.719 (0.637, 2.800) p=1.84e-03	2.058 (0.967, 3.148) p=2.18e-04
Apolipoprotein F	APOF	1.821 (0.673, 2.970) p=1.89e-03	0.918 (-0.213, 2.048) p=1.12e-01
Trefoil factor 2	TFF2	1.723 (0.630, 2.816) p=2.01e-03	2.008 (0.917, 3.100) p=3.13e-04
Tryptophan--tRNA ligase, cytoplasmic	WARS	1.684 (0.608, 2.760) p=2.16e-03	1.464 (0.380, 2.548) p=8.12e-03
Thioredoxin domain-containing protein 5	TXNDC5	1.692 (0.609, 2.775) p=2.20e-03	2.421 (1.335, 3.507) p=1.27e-05
Calsyntenin-3	CLSTN3	1.672 (0.595, 2.749) p=2.36e-03	2.928 (1.856, 4.000) p=8.93e-08
Complement C1q tumor necrosis factor-related protein 1	C1QTNF1	1.661 (0.590, 2.732) p=2.37e-03	2.048 (0.968, 3.127) p=2.02e-04
Fructose-bisphosphate aldolase C	ALDOC	-1.689 (-2.803, -0.575) p=2.97e-03	-2.140 (-3.265, -1.014) p=1.96e-04
Gamma-aminobutyric acid receptor-associated protein-like 1	GABARAPL1	1.673 (0.564, 2.782) p=3.11e-03	3.356 (2.269, 4.443) p=1.52e-09
Transmembrane emp24 domain-containing protein 10	TMED10	1.627 (0.518, 2.736) p=4.04e-03	2.674 (1.572, 3.776) p=2.02e-06
Cystatin-C	CST3	1.683 (0.529, 2.836) p=4.26e-03	3.508 (2.390, 4.626) p=8.25e-10
Collagen alpha-1(XIII) chain	COL13A1	-1.561 (-2.645, -0.478) p=4.73e-03	-2.140 (-3.236, -1.045) p=1.29e-04
Triggering receptor expressed on myeloid cells 2	TREM2	1.606 (0.490, 2.722) p=4.80e-03	1.332 (0.205, 2.458) p=2.06e-02
Lithostathine-1-alpha	REG1A	1.547 (0.451, 2.642) p=5.67e-03	1.868 (0.786, 2.950) p=7.20e-04
RNA-binding protein EWS	EWSR1	1.562 (0.449, 2.674) p=5.94e-03	3.193 (2.103, 4.283) p=9.87e-09
MAM domain-containing glycosylphosphatidylinositol anchor protein 2	MDGA2	-1.495 (-2.577, -0.412) p=6.82e-03	-1.704 (-2.802, -0.606) p=2.35e-03
Alpha-2-HS-glycoprotein	AHSG	-1.479 (-2.575, -0.383) p=8.18e-03	-1.965 (-3.074, -0.855) p=5.22e-04
Insulin-like growth factor-binding protein complex acid labile subunit	IGFALS	-1.518 (-2.647, -0.388) p=8.49e-03	-3.205 (-4.310, -2.100) p=1.39e-08

Model1 adjusted for V5: Age, sex, race SBP, and HR

Model2 adjusted for V5: Age, sex, race, HTN, DM, HF, CHD, AF, SBP, HR, and BMI

- ***Supplemental Table 3:*** *Visit 3 characteristics by incident AV event*

Characteristics at Visit 3	No AV event	AV event	
n	10518	912	P-value
Age (mean (SD))	60 (6)	62 (6)	<0.001
Black (%)	2288 (22)	147 (16)	<0.001
Male (%)	4742 (45)	457 (50)	0.004
DM (%)	1601 (15)	210 (23)	<0.001
HTN (%)	4201 (40)	490 (54)	<0.001
HF (%)	501 (5)	67 (8)	0.001
CHD (%)	741 (7)	92 (10)	0.001
AF (%)	116 (1)	13 (1)	0.471
BMI (mean (SD))	28(6)	30 (6)	<0.001
HR (mean (SD))	66(10)	66(10)	0.956
SBP (mean (SD))	124 (19)	130 (21)	<0.001

- ***Supplemental Table 4:*** *Individual protein aptamer name, HR, CI, p-values from Cox models for association with post-V3 AV events*

Protein name	gene	Model 2 results	Model1 results
Growth/differentiation factor 15	GDF15	1.329 (1.239 - 1.426) p=2.65e-15	1.376 (1.283 - 1.475) p=3.85e-19
Macrophage metalloelastase	MMP12	1.287 (1.203 - 1.376) p=1.91e-13	1.304 (1.220 - 1.393) p=3.66e-15
WAP four-disulfide core domain protein 2	WFDC2	1.230 (1.148 - 1.318) p=4.60e-09	1.194 (1.113 - 1.281) p=6.90e-07
Complement C1q tumor necrosis factor-related protein 1	C1QTNF1	1.180 (1.109 - 1.255) p=1.51e-07	1.175 (1.104 - 1.249) p=3.20e-07
Beta-2-microglobulin	B2M	1.191 (1.113 - 1.275) p=4.09e-07	1.209 (1.131 - 1.293) p=3.18e-08
Insulin-like growth factor-binding protein 2	IGFBP2	1.207 (1.118 - 1.304) p=1.66e-06	1.052 (0.979 - 1.132) p=1.67e-01
Neurocan core protein	NCAN	0.839 (0.781 - 0.901) p=1.67e-06	0.772 (0.722 - 0.826) p=3.97e-14
Afamin	AFM	0.873 (0.824 - 0.924) p=3.73e-06	0.917 (0.862 - 0.976) p=6.37e-03
Monocyte differentiation antigen CD14	CD14.1	1.164 (1.092 - 1.242) p=3.75e-06	1.156 (1.085 - 1.233) p=7.73e-06
Epidermal growth factor receptor	EGFR.1	0.845 (0.787 - 0.908) p=4.29e-06	0.814 (0.759 - 0.873) p=6.91e-09
Thrombospondin-2	THBS2.1	1.162 (1.090 - 1.240) p=4.80e-06	1.246 (1.174 - 1.323) p=6.02e-13
Biotinidase	BTD	0.865 (0.813 - 0.921) p=5.33e-06	0.888 (0.834 - 0.946) p=2.41e-04
Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	SVEP1.1	1.164 (1.089 - 1.244) p=7.43e-06	1.177 (1.101 - 1.257) p=1.50e-06
Secreted and transmembrane protein 1	SECTM1	1.132 (1.071 - 1.196) p=1.14e-05	1.129 (1.069 - 1.193) p=1.64e-05
Protein SET	SET	0.862 (0.806 - 0.922) p=1.35e-05	0.841 (0.787 - 0.900) p=4.40e-07
MAM domain-containing glycosylphosphatidylinositol anchor protein 2	MDGA2	0.860 (0.802 - 0.921) p=1.73e-05	0.842 (0.786 - 0.902) p=1.07e-06
Calsyntenin-3	CLSTN3	1.132 (1.070 - 1.199) p=1.84e-05	1.155 (1.095 - 1.218) p=1.13e-07
Fructose-bisphosphate aldolase C	ALDOC	0.850 (0.789 - 0.916) p=1.94e-05	0.838 (0.776 - 0.905) p=6.15e-06
Immunoglobulin superfamily DCC subclass member 4	IGDCC4	0.861 (0.803 - 0.922) p=1.98e-05	0.812 (0.759 - 0.868) p=1.13e-09
Thioredoxin domain-containing protein 5	TXNDC5	1.117 (1.061 - 1.177) p=2.93e-05	1.117 (1.061 - 1.176) p=2.81e-05
Cystatin-C	CST3	1.166 (1.085 - 1.253) p=2.98e-05	1.196 (1.114 - 1.284) p=8.78e-07
Collagen alpha-1(XIII) chain	COL13A1	0.861 (0.801 - 0.925) p=4.43e-05	0.842 (0.784 - 0.905) p=3.19e-06
Insulin-like growth factor-binding protein complex acid labile subunit	IGFALS	0.878 (0.825 - 0.935) p=4.85e-05	0.819 (0.770 - 0.871) p=1.87e-10
Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	SVEP1	1.147 (1.073 - 1.226) p=5.83e-05	1.157 (1.082 - 1.236) p=2.00e-05
IGF-like family receptor 1	IGFLR1	1.127 (1.063 - 1.196) p=6.83e-05	1.138 (1.076 - 1.203) p=5.43e-06

Ephrin-A4	EFNA4	1.112 (1.053 - 1.174) p=1.18e-04	1.126 (1.069 - 1.185) p=7.23e-06
Gamma-aminobutyric acid receptor-associated protein	GABARAP	1.133 (1.063 - 1.207) p=1.26e-04	1.184 (1.118 - 1.254) p=9.74e-09
Complement component C9	C9	1.144 (1.066 - 1.228) p=1.78e-04	1.136 (1.059 - 1.219) p=3.74e-04
Programmed cell death protein 6	PDCD6	1.123 (1.057 - 1.193) p=1.80e-04	1.100 (1.035 - 1.168) p=2.07e-03
Transmembrane emp24 domain-containing protein 10	TMED10	1.135 (1.062 - 1.214) p=1.92e-04	1.115 (1.042 - 1.193) p=1.62e-03
Angiopoietin-2	ANGPT2	1.144 (1.066 - 1.229) p=2.08e-04	1.231 (1.150 - 1.318) p=2.51e-09
Triggering receptor expressed on myeloid cells 2	TREM2	1.136 (1.059 - 1.218) p=3.51e-04	1.124 (1.048 - 1.205) p=1.11e-03
Leptin	LEP	0.851 (0.778 - 0.930) p=3.98e-04	1.007 (0.930 - 1.091) p=8.63e-01
Gamma-aminobutyric acid receptor-associated protein-like 1	GABARAPL1	1.107 (1.045 - 1.172) p=4.92e-04	1.131 (1.073 - 1.192) p=4.08e-06
Tryptophan--tRNA ligase, cytoplasmic	WARS	1.121 (1.050 - 1.197) p=6.04e-04	1.117 (1.047 - 1.193) p=8.65e-04
Tumor necrosis factor receptor superfamily member 1B	TNFRSF1B.1	1.132 (1.054 - 1.215) p=6.75e-04	1.184 (1.104 - 1.270) p=2.23e-06
Natriuretic peptides B	NPPB.2	1.131 (1.053 - 1.214) p=6.93e-04	1.141 (1.064 - 1.224) p=2.10e-04
Trefoil factor 2	TFF2	1.121 (1.047 - 1.200) p=1.01e-03	1.096 (1.025 - 1.172) p=7.30e-03
Collagen alpha-1(XVIII) chain	COL18A1	1.129 (1.050 - 1.214) p=1.01e-03	1.107 (1.030 - 1.191) p=5.69e-03
Vesicular integral-membrane protein VIP36	LMAN2.1	1.127 (1.049 - 1.210) p=1.12e-03	1.120 (1.041 - 1.204) p=2.25e-03
ADAMTS-like protein 2	ADAMTSL2	1.117 (1.045 - 1.195) p=1.21e-03	1.225 (1.151 - 1.304) p=1.85e-10
Deoxyribonuclease-1-like 2	DNASE1L2	1.095 (1.036 - 1.158) p=1.24e-03	1.129 (1.071 - 1.190) p=6.24e-06
Lithostathine-1-alpha	REG1A	1.114 (1.043 - 1.189) p=1.29e-03	1.088 (1.018 - 1.163) p=1.33e-02
Uronyl 2-sulfotransferase	UST	0.890 (0.828 - 0.956) p=1.45e-03	0.875 (0.815 - 0.939) p=2.30e-04
Growth hormone receptor	GHR	0.886 (0.821 - 0.956) p=1.83e-03	0.968 (0.901 - 1.041) p=3.83e-01
Retinoblastoma-like protein 2	RBL2	1.110 (1.039 - 1.185) p=1.96e-03	1.117 (1.045 - 1.194) p=1.07e-03
WAP four-disulfide core domain protein 1	WFDC1	1.115 (1.040 - 1.196) p=2.22e-03	1.090 (1.016 - 1.169) p=1.63e-02
Serine protease inhibitor Kazal-type 14	SPINK14	1.094 (1.032 - 1.161) p=2.61e-03	1.105 (1.045 - 1.168) p=4.43e-04
RNA-binding protein EWS	EWSR1	1.090 (1.030 - 1.153) p=2.65e-03	1.121 (1.065 - 1.181) p=1.46e-05
Alpha-2-HS-glycoprotein	AHSG	0.903 (0.843 - 0.967) p=3.62e-03	0.899 (0.839 - 0.964) p=2.61e-03
Apolipoprotein F	APOF	1.114 (1.036 - 1.199) p=3.70e-03	1.023 (0.953 - 1.098) p=5.27e-01
Tumor necrosis factor receptor superfamily member 1A	TNFRSF1A	1.114 (1.035 - 1.198) p=3.81e-03	1.175 (1.094 - 1.263) p=1.02e-05

Model1 adjusted for V3: Age, sex, race SBP, and HR
Model2 adjusted for V3: Age, sex, race, HTN, DM, HF, CHD, AF, SBP, HR, and BMI

***-Supplemental Table 5**: Individual protein aptamer name, beta coefficient, CI, p values from linear regression for the association with DI*

Protein name	gene	Model 2 results	Model1 results
Natriuretic peptides B	NPPB.2	-0.021 (-0.025, -0.016) p=1.71e-18	-0.029 (-0.033, -0.024) p=5.04e-38
Angiopoietin-2	ANGPT2	-0.014 (-0.018, -0.009) p=3.73e-10	-0.021 (-0.025, -0.017) p=4.91e-24
Growth hormone receptor	GHR	0.014 (0.009, 0.018) p=1.30e-09	0.013 (0.008, 0.017) p=1.25e-08
Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	SVEP1.1	-0.013 (-0.017, -0.009) p=3.02e-09	-0.020 (-0.024, -0.015) p=8.00e-20
Insulin-like growth factor-binding protein 2	IGFBP2	-0.014 (-0.019, -0.009) p=4.41e-09	-0.011 (-0.015, -0.006) p=1.68e-06
Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	SVEP1	-0.013 (-0.017, -0.009) p=5.96e-09	-0.019 (-0.024, -0.015) p=3.73e-19
Macrophage metalloelastase	MMP12	-0.012 (-0.017, -0.008) p=1.16e-08	-0.017 (-0.021, -0.012) p=1.15e-14
Apolipoprotein F	APOF	-0.010 (-0.014, -0.006) p=5.42e-06	-0.011 (-0.015, -0.007) p=4.19e-07
Deoxyribonuclease-1-like 2	DNASE1L2	-0.009 (-0.013, -0.005) p=9.08e-06	-0.013 (-0.017, -0.009) p=5.34e-10
Vesicular integral-membrane protein VIP36	LMAN2.1	-0.009 (-0.013, -0.005) p=1.27e-05	-0.014 (-0.019, -0.010) p=1.24e-11
Growth/differentiation factor 15	GDF15	-0.009 (-0.014, -0.005) p=4.32e-05	-0.015 (-0.020, -0.011) p=1.99e-12
Protein SET	SET	0.008 (0.004, 0.013) p=9.99e-05	0.014 (0.009, 0.018) p=1.85e-10
Beta-2-microglobulin	B2M	-0.008 (-0.013, -0.004) p=1.32e-04	-0.015 (-0.019, -0.011) p=5.82e-13
Lithostathine-1-alpha	REG1A	-0.008 (-0.012, -0.004) p=1.37e-04	-0.012 (-0.016, -0.008) p=1.21e-08
ADAMTS-like protein 2	ADAMTSL2	-0.008 (-0.012, -0.004) p=1.50e-04	-0.015 (-0.019, -0.011) p=1.49e-13
Insulin-like growth factor-binding protein complex acid labile subunit	IGFALS	0.008 (0.004, 0.012) p=2.28e-04	0.014 (0.010, 0.018) p=1.29e-10
Thrombospondin-2	THBS2.1	-0.008 (-0.012, -0.004) p=2.39e-04	-0.016 (-0.020, -0.012) p=2.25e-14
Collagen alpha-1(XIII) chain	COL13A1	0.007 (0.003, 0.012) p=3.11e-04	0.010 (0.006, 0.014) p=8.68e-07
Complement component C9	C9	-0.007 (-0.011, -0.003) p=3.41e-04	-0.010 (-0.014, -0.006) p=4.06e-06
WAP four-disulfide core domain protein 2	WFDC2	-0.008 (-0.012, -0.004) p=3.55e-04	-0.012 (-0.016, -0.008) p=5.90e-08
Collagen alpha-1(XVIII) chain	COL18A1	-0.008 (-0.012, -0.003) p=3.80e-04	-0.013 (-0.017, -0.009) p=4.26e-09
Cystatin-C	CST3	-0.008 (-0.012, -0.003) p=3.98e-04	-0.015 (-0.020, -0.011) p=1.02e-12
Biotinidase	BTD	0.007 (0.003, 0.011) p=4.10e-04	0.008 (0.004, 0.012) p=4.71e-05
Tumor necrosis factor receptor superfamily member 1B	TNFRSF1B.1	-0.007 (-0.012, -0.003) p=5.15e-04	-0.013 (-0.017, -0.009) p=3.36e-10
IGF-like family receptor 1	IGFLR1	-0.007 (-0.011, -0.003) p=6.52e-04	-0.013 (-0.017, -0.009) p=9.08e-10
Gamma-aminobutyric acid receptor-associated protein	GABARAP	-0.007 (-0.012, -0.003) p=6.59e-04	-0.014 (-0.018, -0.010) p=4.81e-11

Trefoil factor 2	TFF2	-0.007 (-0.011, -0.003) p=7.93e-04	-0.011 (-0.015, -0.006) p=4.97e-07
Uronyl 2-sulfotransferase	UST	0.007 (0.003, 0.011) p=1.15e-03	0.009 (0.005, 0.013) p=2.19e-05
WAP four-disulfide core domain protein 1	WFDC1	-0.007 (-0.011, -0.003) p=1.28e-03	-0.012 (-0.016, -0.008) p=3.21e-08
RNA-binding protein EWS	EWSR1	-0.007 (-0.011, -0.002) p=1.90e-03	-0.013 (-0.017, -0.009) p=3.13e-10
Afamin	AFM	0.007 (0.002, 0.011) p=1.95e-03	0.007 (0.003, 0.011) p=6.75e-04
Thioredoxin domain-containing protein 5	TXNDC5	-0.006 (-0.010, -0.002) p=1.98e-03	-0.010 (-0.014, -0.006) p=1.17e-06
Tryptophan--tRNA ligase, cytoplasmic	WARS	-0.006 (-0.010, -0.002) p=2.03e-03	-0.007 (-0.011, -0.003) p=8.07e-04
Tumor necrosis factor receptor superfamily member 1A	TNFRSF1A	-0.007 (-0.011, -0.002) p=2.37e-03	-0.013 (-0.018, -0.009) p=4.12e-10
Transmembrane emp24 domain-containing protein 10	TMED10	-0.006 (-0.011, -0.002) p=2.75e-03	-0.012 (-0.016, -0.007) p=4.00e-08
Ephrin-A4	EFNA4	-0.006 (-0.010, -0.002) p=3.28e-03	-0.011 (-0.015, -0.007) p=5.26e-08
Epidermal growth factor receptor	EGFR.1	0.006 (0.002, 0.011) p=3.38e-03	0.014 (0.009, 0.018) p=3.29e-10
Monocyte differentiation antigen CD14	CD14.1	-0.006 (-0.010, -0.002) p=4.76e-03	-0.008 (-0.012, -0.003) p=5.00e-04
Fructose-bisphosphate aldolase C	ALDOC	0.006 (0.002, 0.010) p=5.56e-03	0.007 (0.003, 0.011) p=1.36e-03
Programmed cell death protein 6	PDCD6	-0.006 (-0.009, -0.002) p=5.65e-03	-0.004 (-0.008, -0.000) p=3.81e-02
Complement C1q tumor necrosis factor-related protein 1	C1QTNF1	-0.006 (-0.010, -0.002) p=5.67e-03	-0.009 (-0.013, -0.005) p=1.72e-05
Gamma-aminobutyric acid receptor-associated protein-like 1	GABARAPL1	-0.006 (-0.010, -0.001) p=7.70e-03	-0.012 (-0.016, -0.007) p=3.32e-08
Secreted and transmembrane protein 1	SECTM1	-0.005 (-0.009, -0.001) p=1.28e-02	-0.010 (-0.014, -0.006) p=3.95e-06
Neurocan core protein	NCAN	0.005 (0.001, 0.010) p=1.59e-02	0.011 (0.007, 0.016) p=9.83e-08
Triggering receptor expressed on myeloid cells 2	TREM2	-0.005 (-0.009, -0.001) p=1.92e-02	-0.006 (-0.010, -0.002) p=5.01e-03
Retinoblastoma-like protein 2	RBL2	-0.005 (-0.009, -0.001) p=1.94e-02	-0.008 (-0.012, -0.004) p=1.29e-04
MAM domain-containing glycosylphosphatidylinositol anchor protein 2	MDGA2	0.005 (0.001, 0.009) p=2.08e-02	0.006 (0.002, 0.010) p=6.89e-03
Leptin	LEP	0.007 (0.001, 0.013) p=2.08e-02	-0.005 (-0.009, 0.000) p=5.89e-02
Calsyntenin-3	CLSTN3	-0.005 (-0.009, -0.001) p=2.10e-02	-0.010 (-0.014, -0.006) p=2.83e-06
Alpha-2-HS-glycoprotein	AHSG	0.005 (0.001, 0.009) p=2.33e-02	0.007 (0.003, 0.011) p=9.67e-04
Immunoglobulin superfamily DCC subclass member 4	IGDCC4	0.005 (0.001, 0.009) p=2.37e-02	0.009 (0.004, 0.013) p=4.10e-05
Serine protease inhibitor Kazal-type 14	SPINK14	-0.005 (-0.009, -0.001) p=2.41e-02	-0.007 (-0.011, -0.003) p=3.07e-04

Model1 adjusted for V5: Age, sex, race SBP, and HR

Model2 adjusted for V5: Age, sex, race, HTN, DM, HF, CHD, AF, SBP, HR, and BMI

- Supplemental Table 6: *Individual protein aptamer name, odds ratio, CI, p value from Ordinal regression models for association with V7 CT calcification as an ordinary variable (0 calcium group and 3 groups based on the* tertiles of calcium score (4 categories)

Protein name	gene	Model 2 results	Model1 results
Macrophage metalloelastase	MMP12	1.243 (1.181, 1.309) p=1.13e-16	1.212 (1.147, 1.281) p=8.50e-12
Growth/differentiation factor 15	GDF15	1.212 (1.149, 1.278) p=1.29e-12	1.207 (1.144, 1.274) p=9.00e-12
Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	SVEP1	1.196 (1.130, 1.265) p=4.78e-10	1.036 (0.991, 1.083) p=1.23e-01
Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	SVEP1.1	1.189 (1.125, 1.256) p=7.79e-10	1.119 (1.062, 1.178) p=2.36e-05
Retinoblastoma-like protein 2	RBL2	1.134 (1.083, 1.186) p=5.74e-08	1.092 (1.039, 1.147) p=5.15e-04
Serine protease inhibitor Kazal-type 14	SPINK14	1.136 (1.084, 1.190) p=1.09e-07	1.040 (0.992, 1.090) p=1.06e-01
Lithostathine-1-alpha	REG1A	1.136 (1.083, 1.192) p=2.06e-07	1.172 (1.113, 1.233) p=1.27e-09
WAP four-disulfide core domain protein 1	WFDC1	1.141 (1.085, 1.200) p=2.77e-07	1.096 (1.047, 1.148) p=1.02e-04
Insulin-like growth factor-binding protein complex acid labile subunit	IGFALS	0.882 (0.841, 0.926) p=3.70e-07	1.128 (1.076, 1.183) p=6.33e-07
Leptin	LEP	0.830 (0.772, 0.894) p=6.81e-07	1.134 (1.084, 1.186) p=4.19e-08
Thrombospondin-2	THBS2.1	1.139 (1.082, 1.199) p=7.15e-07	0.870 (0.831, 0.911) p=3.70e-09
Growth hormone receptor	GHR	0.878 (0.833, 0.926) p=1.35e-06	0.970 (0.927, 1.015) p=1.84e-01
Apolipoprotein F	APOF	1.134 (1.077, 1.194) p=1.56e-06	1.077 (1.027, 1.129) p=2.40e-03
RNA-binding protein EWS	EWSR1	1.135 (1.077, 1.196) p=2.01e-06	1.078 (1.029, 1.129) p=1.42e-03
Natriuretic peptides B	NPPB.2	1.142 (1.080, 1.208) p=2.83e-06	1.022 (0.977, 1.068) p=3.50e-01
IGF-like family receptor 1	IGFLR1	1.116 (1.066, 1.169) p=3.15e-06	1.005 (0.962, 1.050) p=8.18e-01
Monocyte differentiation antigen CD14	CD14.1	1.112 (1.059, 1.166) p=1.65e-05	1.130 (1.072, 1.191) p=5.02e-06
WAP four-disulfide core domain protein 2	WFDC2	1.121 (1.064, 1.181) p=1.91e-05	1.140 (1.086, 1.197) p=1.31e-07
Collagen alpha-1(XVIII) chain	COL18A1	1.123 (1.065, 1.185) p=2.06e-05	1.099 (1.041, 1.160) p=6.30e-04
Neurocan core protein	NCAN	0.902 (0.858, 0.949) p=5.78e-05	1.108 (1.055, 1.164) p=4.10e-05
Insulin-like growth factor-binding protein 2	IGFBP2	1.116 (1.057, 1.177) p=6.51e-05	1.131 (1.074, 1.192) p=3.86e-06
Trefoil factor 2	TFF2	1.098 (1.046, 1.152) p=1.42e-04	0.879 (0.837, 0.923) p=1.85e-07
Angiopoietin-2	ANGPT2	1.103 (1.049, 1.160) p=1.47e-04	0.932 (0.888, 0.980) p=5.42e-03
Protein SET	SET	0.912 (0.869, 0.957) p=1.66e-04	1.078 (1.031, 1.129) p=1.13e-03
Beta-2-microglobulin	B2M	1.113 (1.052, 1.177) p=1.85e-04	1.180 (1.124, 1.239) p=3.09e-11

Fructose-bisphosphate aldolase C	ALDOC	0.909 (0.864, 0.956) p=1.89e-04	1.139 (1.079, 1.203) p=2.94e-06
Epidermal growth factor receptor	EGFR.1	0.909 (0.864, 0.956) p=2.01e-04	1.009 (0.964, 1.056) p=6.94e-01
Secreted and transmembrane protein 1	SECTM1	1.091 (1.040, 1.143) p=3.10e-04	1.229 (1.168, 1.293) p=2.39e-15
Ephrin-A4	EFNA4	1.086 (1.033, 1.141) p=1.13e-03	1.246 (1.184, 1.311) p=3.53e-17
Triggering receptor expressed on myeloid cells 2	TREM2	1.082 (1.031, 1.135) p=1.35e-03	0.955 (0.912, 0.999) p=4.60e-02
Complement component C9	C9	1.077 (1.029, 1.128) p=1.45e-03	0.891 (0.850, 0.935) p=2.05e-06
Collagen alpha-1(XIII) chain	COL13A1	0.925 (0.882, 0.971) p=1.48e-03	1.014 (0.971, 1.059) p=5.42e-01
Tumor necrosis factor receptor superfamily member 1A	TNFRSF1A	1.084 (1.027, 1.145) p=3.58e-03	1.016 (0.968, 1.066) p=5.12e-01
Vesicular integral-membrane protein VIP36	LMAN2.1	1.081 (1.025, 1.140) p=3.95e-03	1.054 (1.009, 1.100) p=1.82e-02
ADAMTS-like protein 2	ADAMTSL2	1.069 (1.019, 1.121) p=6.49e-03	1.105 (1.055, 1.156) p=1.93e-05
Afamin	AFM	0.940 (0.898, 0.985) p=9.22e-03	1.071 (1.012, 1.134) p=1.70e-02
Tumor necrosis factor receptor superfamily member 1B	TNFRSF1B.1	1.067 (1.014, 1.124) p=1.31e-02	0.916 (0.873, 0.961) p=3.07e-04
Cystatin-C	CST3	1.063 (1.005, 1.124) p=3.38e-02	0.855 (0.816, 0.895) p=3.91e-11
Immunoglobulin superfamily DCC subclass member 4	IGDCC4	0.952 (0.907, 0.999) p=4.68e-02	1.143 (1.093, 1.196) p=7.25e-09
Transmembrane emp24 domain-containing protein 10	TMED10	1.049 (0.990, 1.111) p=1.03e-01	1.151 (1.091, 1.215) p=3.35e-07
Biotinidase	BTD	0.969 (0.926, 1.015) p=1.82e-01	0.981 (0.936, 1.028) p=4.14e-01
Gamma-aminobutyric acid receptor-associated protein	GABARAP	1.032 (0.984, 1.084) p=1.98e-01	1.092 (1.038, 1.148) p=6.13e-04
Tryptophan--tRNA ligase, cytoplasmic	WARS	1.028 (0.982, 1.075) p=2.38e-01	1.039 (0.989, 1.092) p=1.28e-01
Deoxyribonuclease-1-like 2	DNASE1L2	1.025 (0.980, 1.072) p=2.73e-01	1.012 (0.958, 1.069) p=6.71e-01
Thioredoxin domain-containing protein 5	TXNDC5	1.025 (0.979, 1.072) p=2.92e-01	1.146 (1.093, 1.201) p=1.53e-08
Alpha-2-HS-glycoprotein	AHSG	1.018 (0.973, 1.066) p=4.38e-01	1.089 (1.038, 1.142) p=4.37e-04
MAM domain-containing glycosylphosphatidylinositol anchor protein 2	MDGA2	1.016 (0.972, 1.063) p=4.78e-01	1.092 (1.041, 1.145) p=2.98e-04
Uronyl 2-sulfotransferase	UST	1.013 (0.965, 1.063) p=6.00e-01	1.154 (1.098, 1.213) p=1.85e-08
Complement C1q tumor necrosis factor-related protein 1	C1QTNF1	1.013 (0.965, 1.063) p=6.08e-01	1.096 (1.040, 1.155) p=6.17e-04
Calsyntenin-3	CLSTN3	0.989 (0.946, 1.034) p=6.32e-01	0.926 (0.883, 0.971) p=1.47e-03
Programmed cell death protein 6	PDCD6	1.009 (0.966, 1.055) p=6.76e-01	1.022 (0.977, 1.069) p=3.45e-01
Gamma-aminobutyric acid receptor-associated protein-like 1	GABARAPL1	0.999 (0.951, 1.050) p=9.72e-01	0.898 (0.854, 0.944) p=2.49e-05

Model1 adjusted for V5: Age, sex, race SBP, and HR

Model2 adjusted for V5: Age, sex, race, HTN, DM, HF, CHD, AF, SBP, HR, and BMI

- Supplemental Table 7: *Individual protein aptamer name, beta coefficient, CI, p-value from linear regression models for the association with V5 to V7 delta Vmax*

Protein name	gene	Model 2 results	Model1 results
Macrophage metalloelastase	MMP12	2.641 (1.081, 4.201) p=9.16e-04	2.676 (1.134, 4.219) p=6.79e-04
Secreted and transmembrane protein 1	SECTM1	2.520 (0.996, 4.043) p=1.20e-03	2.572 (1.056, 4.088) p=8.93e-04
MAM domain-containing glycosylphosphatidylinositol anchor protein 2	MDGA2	-1.748 (-3.151, -0.346) p=1.46e-02	-1.747 (-3.151, -0.343) p=1.48e-02
IGF-like family receptor 1	IGFLR1	1.574 (0.062, 3.086) p=4.13e-02	1.840 (0.350, 3.330) p=1.55e-02
Growth/differentiation factor 15	GDF15	1.718 (0.049, 3.387) p=4.37e-02	1.789 (0.177, 3.401) p=2.96e-02
Serine protease inhibitor Kazal-type 14	SPINK14	1.591 (0.016, 3.166) p=4.77e-02	1.644 (0.073, 3.214) p=4.02e-02
Natriuretic peptides B	NPPB.2	1.744 (0.013, 3.474) p=4.83e-02	2.181 (0.536, 3.825) p=9.37e-03
Collagen alpha-1(XVIII) chain	COL18A1	1.593 (-0.030, 3.216) p=5.43e-02	1.646 (0.035, 3.257) p=4.53e-02
Angiopoietin-2	ANGPT2	1.549 (-0.061, 3.159) p=5.93e-02	2.131 (0.583, 3.680) p=7.02e-03
Trefoil factor 2	TFF2	1.448 (-0.066, 2.963) p=6.08e-02	1.372 (-0.133, 2.878) p=7.40e-02
Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	SVEP1	1.575 (-0.102, 3.253) p=6.57e-02	1.899 (0.241, 3.557) p=2.48e-02
Alpha-2-HS-glycoprotein	AHSG	1.239 (-0.199, 2.677) p=9.13e-02	1.226 (-0.213, 2.664) p=9.49e-02
Epidermal growth factor receptor	EGFR.1	-1.366 (-2.968, 0.237) p=9.49e-02	-1.725 (-3.273, -0.177) p=2.89e-02
Tumor necrosis factor receptor superfamily member 1B	TNFRSF1B.1	-1.364 (-2.992, 0.263) p=1.00e-01	-0.991 (-2.594, 0.612) p=2.26e-01
RNA-binding protein EWS	EWSR1	-1.404 (-3.083, 0.276) p=1.01e-01	-0.942 (-2.588, 0.704) p=2.62e-01
Beta-2-microglobulin	B2M	1.460 (-0.309, 3.230) p=1.06e-01	1.809 (0.073, 3.544) p=4.11e-02
Vesicular integral-membrane protein VIP36	LMAN2.1	-1.322 (-2.989, 0.344) p=1.20e-01	-1.140 (-2.795, 0.514) p=1.76e-01
Neurocan core protein	NCAN	-1.255 (-2.861, 0.351) p=1.26e-01	-1.542 (-3.019, -0.065) p=4.07e-02
Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	SVEP1.1	1.243 (-0.418, 2.905) p=1.42e-01	1.591 (-0.049, 3.231) p=5.72e-02
Gamma-aminobutyric acid receptor-associated protein	GABARAP	-1.070 (-2.532, 0.392) p=1.51e-01	-0.560 (-1.976, 0.856) p=4.38e-01
Uronyl 2-sulfotransferase	UST	-1.091 (-2.585, 0.404) p=1.53e-01	-1.323 (-2.783, 0.137) p=7.58e-02
Insulin-like growth factor-binding protein 2	IGFBP2	1.167 (-0.487, 2.822) p=1.67e-01	0.623 (-0.933, 2.178) p=4.32e-01
Growth hormone receptor	GHR	-1.141 (-2.783, 0.502) p=1.73e-01	-0.643 (-2.205, 0.919) p=4.19e-01
Tumor necrosis factor receptor superfamily member 1A	TNFRSF1A	-1.136 (-2.823, 0.552) p=1.87e-01	-0.693 (-2.329, 0.943) p=4.06e-01

Biotinidase	BTD	0.895 (-0.494, 2.284) p=2.06e-01	0.878 (-0.510, 2.266) p=2.15e-01
Insulin-like growth factor-binding protein complex acid labile subunit	IGFALS	-0.991 (-2.547, 0.565) p=2.12e-01	-1.311 (-2.812, 0.189) p=8.67e-02
ADAMTS-like protein 2	ADAMTSL2	0.838 (-0.682, 2.358) p=2.80e-01	1.270 (-0.199, 2.738) p=9.01e-02
Lithostathine-1-alpha	REG1A	0.817 (-0.688, 2.323) p=2.87e-01	0.704 (-0.782, 2.190) p=3.53e-01
Thioredoxin domain-containing protein 5	TXNDC5	0.779 (-0.725, 2.283) p=3.10e-01	0.782 (-0.713, 2.277) p=3.05e-01
Monocyte differentiation antigen CD14	CD14.1	0.743 (-0.753, 2.240) p=3.30e-01	0.628 (-0.860, 2.117) p=4.08e-01
Thrombospondin-2	THBS2.1	0.789 (-0.858, 2.436) p=3.48e-01	1.375 (-0.199, 2.948) p=8.68e-02
Immunoglobulin superfamily DCC subclass member 4	IGDCC4	-0.717 (-2.251, 0.818) p=3.60e-01	-1.014 (-2.519, 0.491) p=1.87e-01
Cystatin-C	CST3	0.782 (-0.958, 2.523) p=3.78e-01	1.223 (-0.465, 2.912) p=1.55e-01
Fructose-bisphosphate aldolase C	ALDOC	-0.659 (-2.162, 0.843) p=3.90e-01	-0.667 (-2.163, 0.828) p=3.82e-01
Calsyntenin-3	CLSTN3	-0.642 (-2.112, 0.827) p=3.91e-01	-0.257 (-1.711, 1.196) p=7.29e-01
Apolipoprotein F	APOF	0.617 (-0.927, 2.161) p=4.33e-01	0.404 (-1.106, 1.915) p=6.00e-01
Programmed cell death protein 6	PDCD6	-0.515 (-1.894, 0.864) p=4.64e-01	-0.599 (-1.976, 0.779) p=3.94e-01
Leptin	LEP	-0.836 (-3.114, 1.441) p=4.72e-01	0.515 (-1.238, 2.268) p=5.64e-01
Triggering receptor expressed on myeloid cells 2	TREM2	0.508 (-0.961, 1.977) p=4.98e-01	0.489 (-0.979, 1.957) p=5.14e-01
Gamma-aminobutyric acid receptor-associated protein-like 1	GABARAPL1	-0.508 (-2.017, 1.001) p=5.09e-01	-0.060 (-1.534, 1.414) p=9.36e-01
Ephrin-A4	EFNA4	0.472 (-1.155, 2.100) p=5.69e-01	0.660 (-0.947, 2.266) p=4.21e-01
Deoxyribonuclease-1-like 2	DNASE1L2	-0.340 (-1.776, 1.097) p=6.43e-01	0.070 (-1.348, 1.488) p=9.23e-01
WAP four-disulfide core domain protein 2	WFDC2	0.389 (-1.271, 2.049) p=6.46e-01	0.414 (-1.238, 2.066) p=6.23e-01
Transmembrane emp24 domain-containing protein 10	TMED10	-0.367 (-2.101, 1.367) p=6.78e-01	-0.097 (-1.817, 1.623) p=9.12e-01
Complement component C9	C9	0.264 (-1.108, 1.636) p=7.06e-01	0.377 (-0.995, 1.749) p=5.90e-01
Protein SET	SET	-0.285 (-1.816, 1.247) p=7.15e-01	-0.628 (-2.134, 0.878) p=4.14e-01
Afamin	AFM	0.234 (-1.223, 1.690) p=7.53e-01	0.356 (-1.084, 1.795) p=6.28e-01
Retinoblastoma-like protein 2	RBL2	0.223 (-1.185, 1.630) p=7.56e-01	0.343 (-1.063, 1.748) p=6.33e-01
Tryptophan--tRNA ligase, cytoplasmic	WARS	0.061 (-1.386, 1.508) p=9.34e-01	0.271 (-1.167, 1.710) p=7.12e-01
WAP four-disulfide core domain protein 1	WFDC1	-0.061 (-1.617, 1.496) p=9.39e-01	0.057 (-1.490, 1.604) p=9.42e-01
Collagen alpha-1(XIII) chain	COL13A1	0.031 (-1.406, 1.467) p=9.67e-01	-0.152 (-1.586, 1.282) p=8.36e-01
Complement C1q tumor necrosis factor-related protein 1	C1QTNF1	-0.008 (-1.513, 1.497) p=9.92e-01	0.032 (-1.472, 1.535) p=9.67e-01

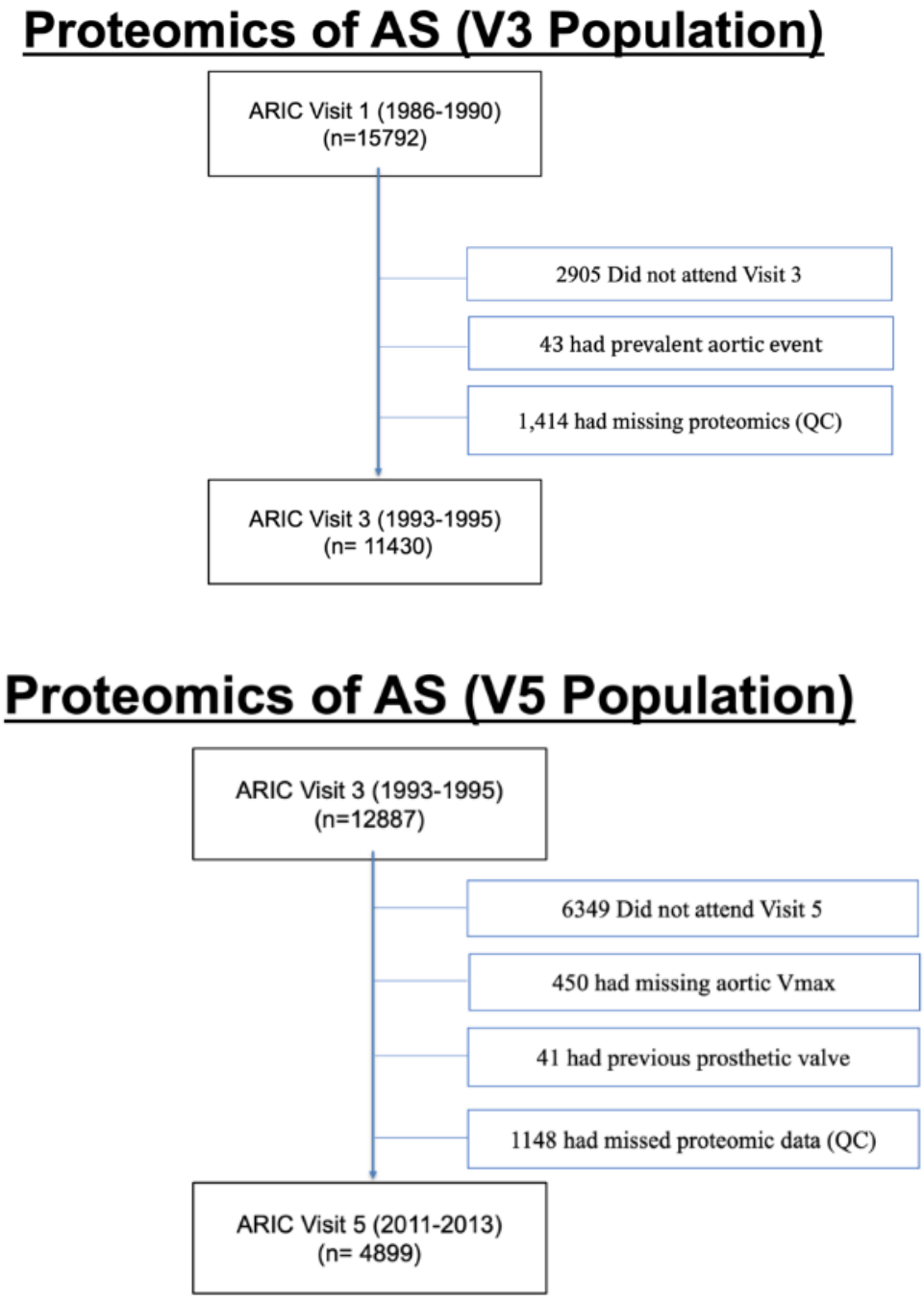
Model1 adjusted for V5: Age, sex, race SBP, and HR

Model2 adjusted for V5: Age, sex, race, HTN, DM, HF, CHD, AF, SBP, HR, BMI and, V7 SBP, V7 HR

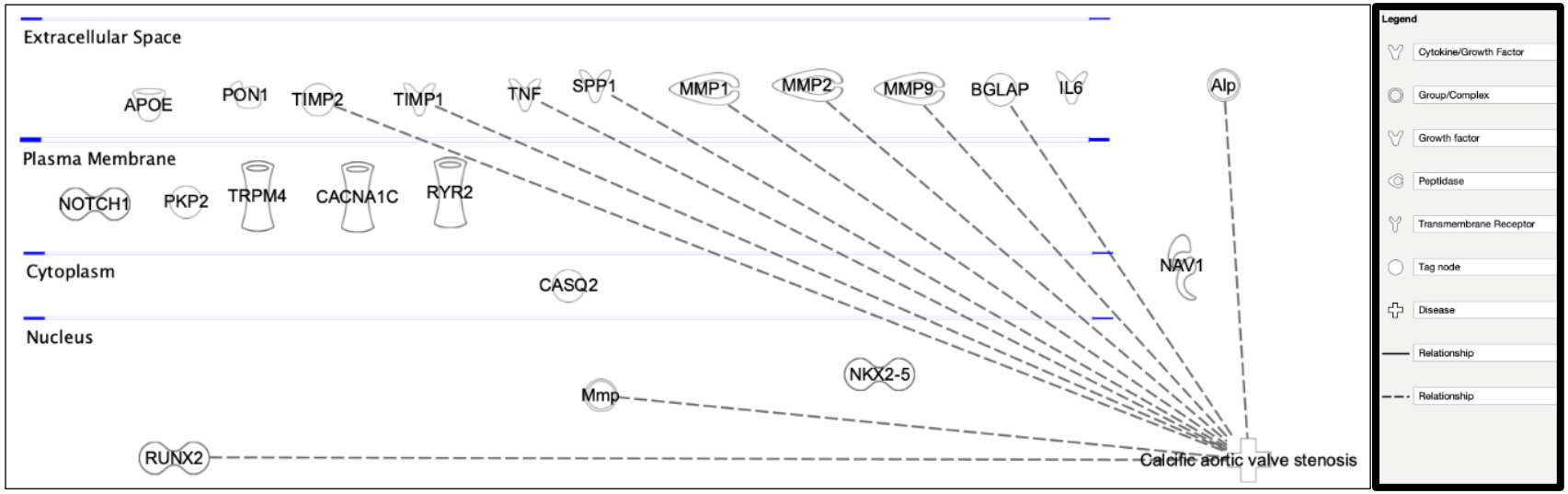
- Supplemental Table 8: *The Highly interconnected proteins (proteins networks) and their related Diseases and functions.* Networks are scored according to the connections with the 52 focus molecules and sorted based on the score

Top Diseases and Functions	**Molecules in Network**	**Score**	**Focus Molecules**
[Connective Tissue Development and Function, Embryonic Development, Skeletal and Muscular System Development and Function]	ADRB, AHSG, Angiotensin II receptor type 1, ANGPT2, C/EBP, CaMKII, CLSTN3, COL13A1, collagen, Collagen type IV, Cyclin D,ERK1/2,estrogen receptor,GDF15,GHR,Growth hormone,Igf,IGFALS,IGFBP2,IL1,Integrin,JINK1/2,LEP,Mmp,NADPH oxidase,NFkB (family),NPPB,PDCD6,Pro-inflammatory Cytokine,Secretase gamma,TFF2,THBS2,TMED10,Tnf (family),TREM2	34	15
[Cellular Development, Cellular Growth and Proliferation, Organ Development]	ADAMTSL2,ALDOC,AQP11,asparagine,ATG12/ATG5/ATG16L1,BTD,CDKN1B,DNAH7,DNASE1L2,Eda, EGFR,Egfr-Erbb2,EGFR/PDGFR/IGFR,ER-α-Estradiol,EWSR1,GABARAP,GABARAPL1,GOT,IL6,INHA,Jnk,KIF5B-RET,MDGA2,MTORC1,neopterin,PI3K p85,PLEK2,RASGEF1B,REG1A,ribose,SRC (family),SVEP1,TGFB1,Tpsab1,UST	26	12
[Lipid Metabolism, Ophthalmic Disease, Organismal Injury and Abnormalities]	26s Proteasome,AFM,Akt,Alpha catenin,APOF,C9,caspase,CD14,COL18A1,Collagen Alpha1,Collagen type I (complex),Collagen(s),Creb,cytokine,Focal adhesion kinase,HDL,Hsp70,Hsp90,Ifn,IgG,IL12 (complex),Immunoglobulin,Laminin (complex),LDL,MMP12,NCAN,Nos,p70 S6k,PDGF BB,PI3K (family),Pka,PP2A,SECTM1,STAT5a/b,Tgf beta	16	8
[Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking]	11,12-epoxyeicosatrienoic acid,ADAM8,Adaptor,CCL15,DGKA,DTYMK,EFNA4,elovanoid N32,elovanoid N34,ETV5,GATM,GPBAR1,GPM6B,ICAM1,IGDCC4,IGF receptor,IGFLR1,IL4,IL5RA,indican,LMAN2,LTC4S,mannose,MTMR8,Pik3r,PRDM10,REL,Serpina3g (includes others),SET,thymidine,TNFRSF6B,TP53,TXNDC5,WFDC1,WFDC2	16	8
[Inflammatory Disease, Ophthalmic Disease, Organismal Injury and Abnormalities]	ALT,Ap1,B2M,C1QTNF1,CG,CST3,Cyclin A,ERK,FSH,Gsk3,Histone h3,Histone h4,Ige,IgG1,Igm,Insulin,Interferon alpha,Lh,Mapk,Mek,NFkB (complex),Notch,P38 MAPK,p85 (pik3r),PI3K (complex),Pkc(s),Rac,RBL2,RNA polymerase II, STAT,TCR,TNFRSF1A,TNFRSF1B,Vegf,WARS1	13	7

Supplemental Figure1(consort diagrams)



Supplemental Figure2*. Calcific Aortic Valve Stenosis Disease Ingenuity Network showing 22 molecules that were found to be associated with AS based on IPA databases and*



Apolipoprotein E (**APOE**), Paraoxonase 1 (**PON1**), Tissue inhibitor of metalloproteinase 2 (**TIMP2**), Tissue inhibitor of metalloproteinase 1 (**TIMP1**), Tumor necrosis factor (**TNF),** Secreted Phosphoprotein 1 (**SPP1**), Matrix Metallopeptidase 1 (**MMP1**), Matrix Metallopeptidase 2 (**MMP2**), Matrix Metallopeptidase 9 (**MMP9**), Bone Gamma-Carboxyglutamate Protein (**BGLAP**), Interleukin 6 (**IL6**), A**lkaline phosphatase** (**ALP**), Notch Receptor 1**(NOTCH1),** plakophilin-2 (**PKP2**), transient receptor potential cation channel subfamily M member 4 (**TRPM4**), Calcium Voltage-Gated Channel Subunit Alpha1 C **(CACNA1C**), **RYR2** (Ryanodine Receptor 2), Calsequestrin 2 (**CASQ2**), neuron navigator 1 (**NAV1**), Runt-related transcription factor 2 (**RUNX2**), Homeobox protein Nkx-2.5 **(NKX2-5**).

Summary of Conclusions:

In a diverse community-based cohort of older adults:

Subclinical VHD is common, with 39% at risk of VHD (Stage A) and 17% with progressive VHD (Stage B). Stage A and Stage B VHD are associated with a heightened risk of incident cardiovascular events independent of traditional cardiovascular risk factors. VHD stages progress over six years in late life, with a several-fold increase in the prevalence of severe VHD (Stage C/D). These findings clarify the burden of VHD in late-life and highlight the public health importance of interventions to mitigate VHD progression.

We identified 38 circulating proteins with robust associations with AV hemodynamics, calcification, and risk of incident AV-related hospitalizations. Higher MMP12 values demonstrated particularly robust and consistent associations with worse AV hemodynamics, progression in AV over time, magnitude of AV calcification, and risk of incident AV events. Pathway analysis identified IFNG as a potentially important upstream regulator related to 9 of 38 identified proteins, including MMP12. These findings highlight a potential novel biomarker for AS risk, and a novel putative targetable pathway to prevent AS progression.

Discussion and perspectives:

In addition to the ARIC study's diversity and large sample size, it is characterized by being a thoroughly phenotyped longitudinal community-based cohort. Thus, because of the availability of adjudicated cardiovascular events, active event surveillance, repeated cardiac echocardiography assessments, repeated serum proteomics, cardiac CT, and critical clinical covariates. As a result, this project is one of the first to quantify the prevalence, prognostic relevance, and progression of ACC/AHA VHD stages and introduce evolving biomarkers and potential new drugs targets for AS.

However, the generalizability of our results is limited by the absence of an external validation analysis and by the non-attendance of surviving ARIC participants at Visit 5. Also, the internal validity is affected by the observational nature of ARIC. So, we cannot conclude causal relationships. Using ICD codes to detect VHD-related hospitalization and interventions and the absence of quantitative assessments for some valvular lesions and the serum proteins may be sources of measurement bias.

Our results encourage giving more attention to preventing the progression of VHD in older adults, particularly AS. Future programs dedicated to screening and controlling the VHD-associated risk factors are needed. Furthermore, targeted biomarkers studies for evolving serum biomarkers like MMP12 are required in order to validate its role in early AS detection and monitoring its progression. Also, new therapeutic targets are proposed to be studied through the different phases of randomized clinical trials. The availability of a biomarker for the proposed new drug effect will be an excellent opportunity to facilitate the discovery phase.