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Systemic Proinflammatory–Profibrotic Response in Aortic Stenosis Patients with Diabetes and its Relationship with Myocardial Remodeling and Clinical Outcome

대동맥판막협착증 환자에서 당뇨에 의한 혈장단백체의 변화 및 심근의 재형성과 예후와의 관련성 연구

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Abstract

Systemic Proinflammatory–Profibrotic Response in Aortic Stenosis Patients with Diabetes and its Relationship with Myocardial Remodeling and Clinical Outcome

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Background: It is unclear whether and how diabetes mellitus may aggravate myocardial fibrosis and remodeling in the pressure-overloaded heart. We investigated the impact of diabetes on the prognosis of aortic stenosis (AS) patients and its underlying mechanisms using comprehensive noninvasive imaging studies and plasma proteomics.

Methods: Severe AS patients undergoing both echocardiography and cardiovascular magnetic resonance (CMR) (n=253 of which 66 had diabetes) comprised the imaging cohort. The degree of replacement and diffuse interstitial fibrosis by late gadolinium enhancement (LGE) and extracellular volume fraction (ECV) was quantified using CMR. Plasma samples were analyzed with the multiplex proximity extension assay for 92 proteomic biomarkers in a separate biomarker cohort of severe AS patients (n=100 of which 27 had diabetes).

Results: In the imaging cohort, diabetic patients were older (70.4±6.8 vs. 66.7±10.1 years) and had a higher prevalence of ischemic heart disease (28.8% vs. 9.1%), with more advanced ventricular diastolic dysfunction. On CMR, diabetic patients had increased replacement and diffuse interstitial fibrosis (LGE% 0.3

[0.0−1.6] vs. 0.0 [0.0−0.5], p=0.009; ECV% 27.9 [25.7−30.1] vs. 26.7 [24.9−28.5], p=0.025). Plasma proteomics analysis of the biomarker cohort revealed that 9 proteins (E−selectin, interleukin−1 receptor type 1, interleukin−1 receptor type 2, galectin−4, intercellular adhesion molecule 2, integrin beta−2, galectin−3, growth differentiation factor 15, and cathepsin D) are significantly elevated in diabetic AS patients. Pathway over−representation analyses of the plasma proteomics with Gene Ontology terms indicated that pathways related to inflammatory response and extracellular matrix components were enriched, suggesting that diabetes is associated with systemic effects that evoke proinflammatory and profibrotic response to the pressure−overloaded myocardium. During follow−up (median 6.3 years [IQR 5.2−7.2]) of the imaging cohort, 232 patients received aortic valve replacement (AVR) with 53 unexpected heart failure admissions or death. Diabetes was a significant predictor of heart failure and death, independent of clinical covariates and AVR (hazard ratio 1.88, 95% confidence interval 1.06−3.31, p=0.030).

Conclusion: Plasma proteomic analyses indicate that diabetes potentiates the systemic proinflammatory and profibrotic milieu in AS patients. These systemic biological changes underlie the increase of myocardial fibrosis, diastolic dysfunction, and worse clinical outcomes in severe AS patients with concomitant diabetes.

Keyword: aortic valve stenosis, diabetes mellitus, magnetic resonance imaging, echocardiography, proteome

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Chapter 1. Introduction

1.1. Study background

Aortic stenosis (AS) is initially a disease of the heart valve but its prognosis depends greatly on the health of the myocardium. Sustained pressure overload by AS induces ventricular hypertrophy and myocardial fibrosis that leads to ventricular decompensation, initially diastolic dysfunction and later, systolic dysfunction.(1, 2)

Myocardial fibrosis is commonly observed in two forms, diffuse interstitial and focal replacement fibrosis. Both forms of fibrosis can be imaged noninvasively with cardiac magnetic resonance (CMR) with gadolinium-based contrast agents: diffuse interstitial fibrosis is quantified by extracellular volume fraction (ECV) on T1 mapping and replacement fibrosis by late gadolinium enhancement (LGE).(3) The former is partially reversible while the latter remains even after relief of pressure overload by aortic valve replacement (AVR) in AS.(4, 5) Both increased ECV and LGE are associated with worse prognosis in patients with AS.(6-8)

Diabetes mellitus is a systemic disease that affects the myocardium directly. 'Diabetic cardiomyopathy' was first described from autopsies of diabetic patients who manifested with heart failure but no evidence of coronary problems, valvular disease or hypertension.(9) Subsequent investigations have demonstrated that diabetic patients have increased myocardial fibrosis which may be explained by multiple biological and molecular mechanisms.(10,

11)

1.2. Purpose of research

Most studies on the interaction of diabetes with AS have focused on the progression of valvular stenosis.(12-15) There have been only few studies that addresses the impact of diabetes in AS patients, especially, how it is related to myocardial health.(16-18) Considering that diabetes is associated with worse prognosis in AS patients,(19, 20) we hypothesized that diabetes would aggravate the degree of myocardial fibrosis in AS patients. The objective of this study was two-fold; first, to elucidate the prognostic impact of diabetes in AS patients and second, to dissect its underlying mechanisms using comprehensive noninvasive imaging and plasma proteomics.

Chapter 2. Methods

2.1. Study Population

This study utilized two prospectively enrolled cohorts of AS patients: the imaging cohort for the assessment of the myocardial health using CMR and echocardiography with long term follow-up for clinical events, and a biomarker cohort for the assessment of enriched circulating proteins using multiplex proximity extension assay.

The imaging cohort consisted of 253 patients with moderate or severe AS prospectively enrolled mainly from 2011 to 2015 at three tertiary medical centers in Korea (Seoul National University Hospital [n=146], Asan Medical Center [n=40], and Samsung Medical Center [n=66]). All participants in this cohort underwent comprehensive echocardiography and CMR; patients with estimated glomerular filtration rate <30 ml/min/1.73m^2 were excluded, considering the eligibility for CMR. The biomarker cohort consisted of 100 patients with severe AS undergoing surgical AVR enrolled prospectively from 2018 to 2021 at Seoul National University Hospital (Figure 1). Detailed inclusion and exclusion criteria are in described in the following subsections.

Two separate cohorts were used in the study because patients in the imaging cohort did not undergo blood sample collection and most of the patients in the biomarker cohort did not undergo CMR and were followed for less than one year (median follow-up 6.6

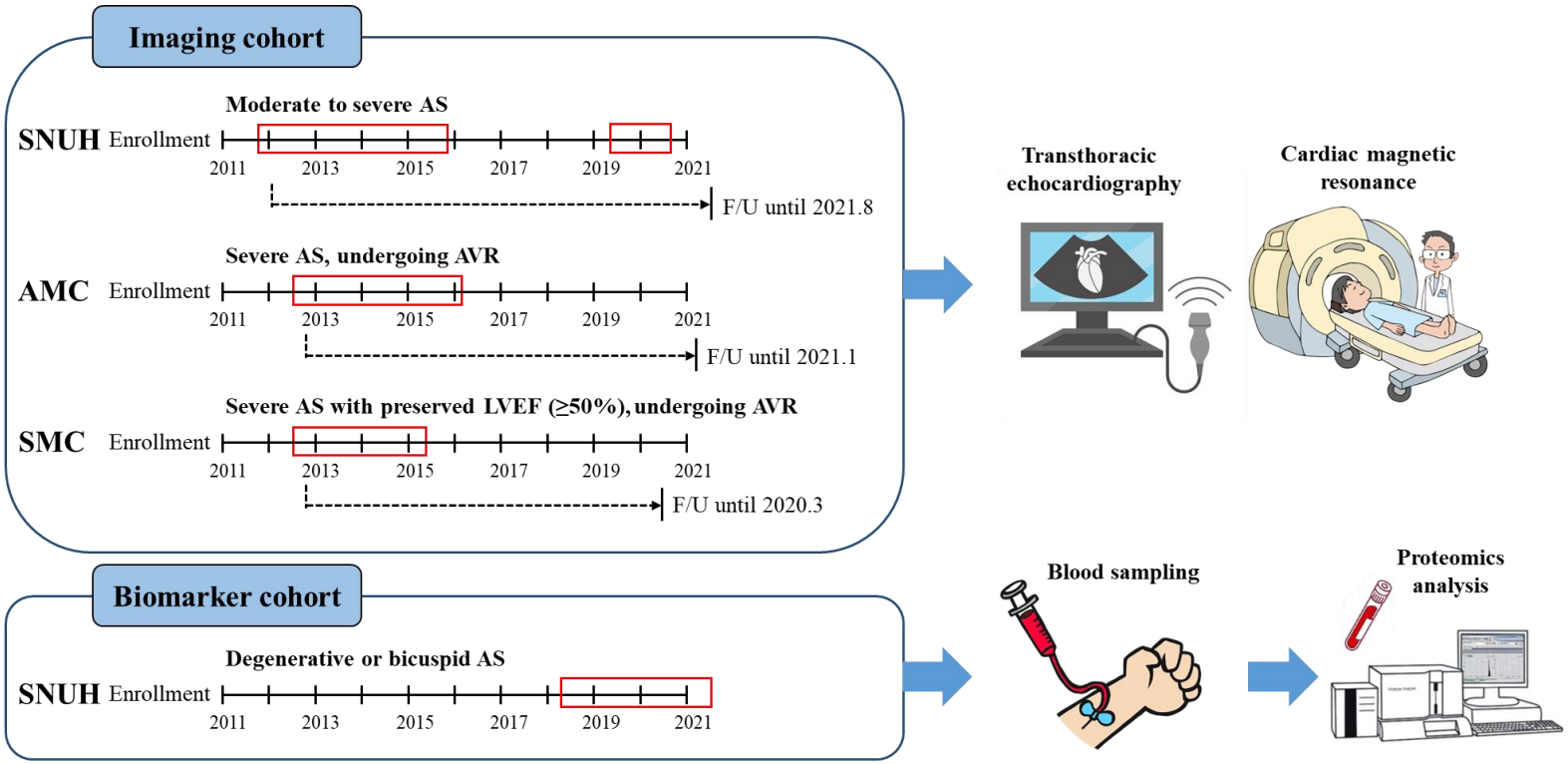


Figure 1. Schematic diagram of study population.

AMC, Asan Medical Center; AS, aortic stenosis; AVR, aortic valve replacement; F/U, follow-up; LVEF, left ventricular ejection fraction; SMC, Samsung Medical Center; SNUH, Seoul National University Hospital.

[IQR 3.9–12.2] months). Both cohorts were approved by the institutional review boards of the three institutions and all study subjects gave written informed consent before enrollment.

Patients who were being treated for diabetes and those who were newly diagnosed with diabetes during the initial evaluation were classified as the diabetic group and the medication status at enrollment was assessed. Ischemic heart disease (IHD) was defined as prior coronary intervention or concomitant coronary artery bypass grafting performed together with AVR.

2.1.1. Study enrollment criteria and follow–up for the imaging cohort

Consecutive patients with moderate or severe aortic stenosis (AS) were enrolled prospectively from three large–volume tertiary medical centers in Korea (Seoul National University Hospital [n=146], Asan Medical Center [n=40], and Samsung Medical Center [n=66]). All patients underwent cardiac magnetic resonance (CMR) imaging with T1 mapping performed both before and following intravenous gadolinium contrast administration.

In Seoul National University Hospital, patients with moderate or severe AS were enrolled prospectively from October 2011 to November 2015 (n=126), and from April 2019 to August 2020 (n=20). The enrollment criteria were moderate or severe AS defined by echocardiography as transaortic peak velocity ≥3.0 m/s or transaortic mean pressure gradient ≥30 mmHg, and aortic valve area of <1.5 cm2. For patients with left ventricular ejection fraction <40%, only the criteria of aortic valve area of <1.5 cm2 used. The

exclusion criteria were concomitant valvular disease of at least moderate severity other than AS or non-cardiac comorbid conditions of life expectancy <1 year, serum creatinine >2.0 mg/dL or calculated creatinine clearance <30 ml/min/1.73m2, presence of artificial cochlear or permanent pacemaker, previous history of significant side effects after magnetic resonance imaging, chronic treatment with oral, intravenous, or intra-articular corticosteroids, untreated hyperthyroidism or hypothyroidism with thyroid-stimulating hormone levels more than 2 times upper limit of normal, women who are pregnant or breast-feeding, and history of chronic obstructive pulmonary disease or asthma on bronchodilators including long-acting beta2-agonist, anticholinergics, or inhaled steroids or recent acute aggravation of chronic obstructive pulmonary disease in the past 6 months. Last clinical follow-up or death was checked on August 13th, 2021.

In Asan Medical Center, patients with severe AS according to the guidelines awaiting aortic valve replacement were enrolled from June 2012 to January 2016 (n=40). Exclusion criteria were the presence of an implantable cardiac device, advanced renal dysfunction (estimated glomerular filtration rate <30 ml/min/1.73m^2), previous valve replacement, and presence of another coexistent myocardial pathology such as cardiac amyloidosis, hypertrophic cardiomyopathy, or myocarditis. Last clinical follow-up or death was checked on January 18th, 2021.

In Samsung Medical Center, patients with severe AS with preserved systolic function awaiting aortic valve replacement were

enrolled from June 2012 to March 2015 (n=66). The enrollment criteria were severe AS with preserved systolic function defined as indexed aortic valve area (AVA) <0.6 cm^2/m^2 and left ventricular ejection fraction ≥50%. The exclusion criteria were concomitant valvular disease of at least moderate severity other than AS, previous aortic valve replacement, obstructive epicardial coronary artery disease (>30% luminal stenosis in at least 1 coronary artery on coronary angiography), history of myocardial infarction or acute coronary syndrome; any absolute contraindication to CMR, or estimated glomerular filtration rate <30 ml/min/1.73m^2. Last clinical follow-up or death was checked on March 1st, 2020.

2.1.2. Study enrollment criteria and sample collection for the biomarker cohort

In Seoul National University Hospital, patients with severe AS undergoing aortic valve replacement were enrolled prospectively from March 2018 to June 2021 (n=100). After informed consent, 10 cc of patient blood was collected in EDTA-coated tubes and separated into plasma and buffy coat layers by centrifugation. The plasma and buffy coat samples were stored in EDTA-coated tubes in a deep freezer at −80℃, and the plasma samples were used for this study. The enrollment criteria were degenerative or bicuspid AS diagnosed on echocardiography, and exclusion criteria were AS due to rheumatic valvular heart disease, endocarditis or congenital valvular heart disease other than bicuspid AV.

2.2. Echocardiographic evaluation

Echocardiography was performed using commercially available machines and the severity of AS was determined according to the contemporary guidelines.(21) The left ventricular (LV) chamber size, and systolic and diastolic function were also evaluated and categorized according to the most updated guidelines.(22, 23)

2.3. Cardiac magnetic resonance analysis

In the imaging cohort, CMR was performed at a median interval of 11 [2–29] days from the echocardiography. The CMR images were obtained using either 1.5–T or 3–T scanners. The details of the scanners, field strengths, T1 mapping sequences, contrast agents, and summary of imaging analyses for each study center are presented in Table 1.(8) Briefly, CMR scans consisted of balanced steady–state free precession cine images, pre– and post–gadolinium T1 mapping, and the LGE images. The chamber sizes and myocardial mass were quantified according to a standardized protocol.(7)

T1 values were measured in pre– and post–gadolinium T1 maps from manually drawn regions of interest at the short–axis mid–ventricular septum and blood pool, with manual offsetting from the endocardial and epicardial borders to minimize partial voluming.(24) Infarct–related LGE was excluded while non–infarct LGE was included in the region of interest.(7, 25)

Table 1. Technical details of cardiovascular magnetic resonance by study centers.

Site	Scanner	Pulse sequence (pre)	Pulse sequence (post)	Contrast agent, dose and timing	N	Mean native T1, ms	Mean ECV%	Mean LGE%
Seoul National University Hospital, Seoul, Korea	Siemens Trio 3T	MOLLI 3(3)–3(3)–5	MOLLI 3(3)–3(3)–5	Magnevist 0.20 mmol/kg 10 mins	125	1232±53	27.9±3.3	1.0±2.8
Seoul National University Hospital, Seoul, Korea	Siemens Skyra 3T	MOLLI 3(3)–3(3)–5	MOLLI 3(3)–3(3)–5	Dotarem 0.20 mmol/kg 15 mins	21	1271±59	28.3±3.7	0.7±0.6

Asan Medical Center, Seoul, Korea	Siemens Avanto 1.5T	MOLLI 3(3)-3(3)-5	MOLLI 3(3)-3(3)-5	Gadovist 0.10 mmol/kg 20 mins	40	1000±39	26.3±2.3	0.5±1.7
Samsung Medical Center, Seoul, Korea	Siemens Avanto 1.5T	MOLLI 5(3)-3	MOLLI 4(1)-3(1)-2	Gadobutrol 0.10 mmol/kg 15 mins	66	992±60	26.3±2.4	1.5±3.1

ECV, extracellular volume fraction; LGE, late gadolinium enhancement; MOLLI, Modified Look-Locker inversion recovery

The mid-ventricular septum was chosen for analysis as it correlates well with analysis of the entire 17 myocardial segments, is simpler to perform, and can avoid partial volume effects from apical segments.(26) Moreover, measurement from regions of interest drawn at the mid-ventricular septum as opposed to the whole mid-ventricular myocardium has shown improved reproducibility.(27) Guidelines also recommend measurement from a single region of interest drawn at the short-axis mid-ventricular septum for global assessment of diffuse disease.(25) The degree of diffuse interstitial fibrosis was assessed by calculating ECV from the pre- and post-gadolinium T1 values at the mid-ventricular septum and blood pool, and hematocrit from blood samples at the time of CMR.(2, 3)

The presence of LGE was assessed visually on short-axis images acquired by phase sensitive inversion recovery sequence by two independent experienced personnel. LGE quantification with the 5-SD technique was performed using CVI42 (Circle Cardiovascular Imaging Inc., Calgary, Canada). The regions of interest for LGE were drawn semi-automatically as pixels of the myocardium with a signal intensity >5 standard deviations of the normal remote myocardium, and the LGE% was calculated by dividing the LGE area by the total LV myocardial area.(28) Areas of signal contamination by epicardial fat or blood pool were manually excluded.

2.4. Plasma proteomics assay

Blood samples from the biomarker cohort were collected preoperatively in EDTA bottles, divided into plasma and buffy coat layers with centrifugation, and then stored at −80℃. For plasma proteomics analysis, deep frozen plasma samples were shipped to Olink Proteomics (Uppsala, Sweden) and the plasma levels of 92 protein biomarkers were measured using commercially available multiplex proximity extension assay kits (Olink Cardiovascular III Panel, Table 2). This high−throughput technique utilizes immunoassay with oligonucleotide−labeled antibodies followed by real−time polymerase chain reaction for simultaneous quantification of target proteins with high specificity and scalability.(29) After normalization procedures, plasma levels were expressed for each protein in relative quantification units called normalized protein expression (NPX) using the Log_2 scale (1 NPX difference equaling 2−fold change in protein concentration).

Table 2. List of protein biomarkers included in Cardiovascular Panel III v.6114 (Olink, Uppsala, Sweden).

Target	Abbreviation	UniProt ID
Aminopeptidase N	AP-N	P15144
Azurocidin	AZU1	P20160
Bleomycin hydrolase	BLM hydrolase	Q13867
Cadherin-5	CDH5	P33151
Carboxypeptidase A1	CPA1	P15085
Carboxypeptidase B	CPB1	P15086
Caspase-3	CASP-3	P42574
Cathepsin D	CTSD	P07339
Cathepsin Z	CTSZ	Q9UBR2
C-C motif chemokine 15	CCL15	Q16663
C-C motif chemokine 16	CCL16	O15467
C-C motif chemokine 24	CCL24	O00175
CD166 antigen	ALCAM	Q13740
Chitinase-3-like protein 1	CHI3L1	P36222
Chitotriosidase-1	CHIT1	Q13231
Collagen alpha-1(I) chain	COL1A1	P02452
Complement component C1q receptor	CD93	Q9NPY3
Contactin-1	CNTN1	Q12860
C-X-C motif chemokine 16	CXCL16	Q9H2A7
Cystatin-B	CSTB	P04080
Elafin	PI3	P19957

Ephrin type-B receptor 4	EPHB4	P54760
Epidermal growth factor receptor	EGFR	P00533
Epithelial cell adhesion molecule	Ep-CAM	P16422
E-selectin	SELE	P16581
Fatty acid-binding protein, adipocyte	FABP4	P15090
Galectin-3	Gal-3	P17931
Galectin-4	Gal-4	P56470
Granulins	GRN	P28799
Growth/differentiation factor 15	GDF-15	Q99988
Insulin-like growth factor-binding protein 1	IGFBP-1	P08833
Insulin-like growth factor-binding protein 2	IGFBP-2	P18065
Insulin-like growth factor-binding protein 7	IGFBP-7	Q16270
Integrin beta-2	ITGB2	P05107
Intercellular adhesion molecule	ICAM-2	P13598
Interleukin-1 receptor type 1	IL-1RT1	P14778
Interleukin-1 receptor type 2	IL-1RT2	P27930
Interleukin-17 receptor A	IL-17RA	Q96F46
Interleukin-18-binding protein	IL-18BP	O95998
Interleukin-2 receptor subunit alpha	IL2-RA	P01589
Interleukin-6 receptor subunit alpha	IL-6RA	P08887
Junctional adhesion molecule A	JAM-A	Q9Y624
Kallikrein-6	KLK6	Q92876

Low-density lipoprotein receptor	LDL receptor	P01130
Lymphotoxin-beta receptor	LTBR	P36941
Matrix extracellular phosphoglycoprotein	MEPE	Q9NQ76
Matrix metalloproteinase-2	MMP-2	P08253
Matrix metalloproteinase-3	MMP-3	P08254
Matrix metalloproteinase-9	MMP-9	P14780
Metalloproteinase inhibitor 4	TIMP4	Q99727
Monocyte chemotactic protein 1	MCP-1	P13500
Myeloblastin	PRTN3	P24158
Myeloperoxidase	MPO	P05164
Myoglobin	MB	P02144
Neurogenic locus notch homolog protein 3	Notch 3	Q9UM47
N-terminal prohormone brain natriuretic peptide	NT-proBNP	N/A
Osteopontin	OPN	P10451
Osteoprotegerin	OPG	O00300
Paraoxonase	PON3	Q15166
Peptidoglycan recognition protein 1	PGLYRP1	O75594
Perlecan	PLC	P98160
Plasminogen activator inhibitor 1	PAI	P05121
Platelet endothelial cell adhesion molecule	PECAM-1	P16284
Platelet glycoprotein VI	GP6	Q9HCN6

Platelet-derived growth factor subunit A	PDGF subunit A	P04085
Proprotein convertase subtilisin/kexin type 9	PCSK9	Q8NBP7
Protein delta homolog 1	DLK-1	P80370
P-selectin	SELP	P16109
Pulmonary surfactant-associated protein D	PSP-D	P35247
Resistin	RETN	Q9HD89
Retinoic acid receptor responder protein 2	RARRES2	Q99969
Scavenger receptor cysteine-rich type protein M130	CD163	Q86VB7
Secretoglobin family 3A member 2	SCGB3A2	Q96PL1
Spondin-1	SPON1	Q9HCB6
ST2 protein	ST2	Q01638
Tartrate-resistant acid phosphatase type 5	TR-AP	P13686
Tissue factor pathway inhibitor	TFPI	P10646
Tissue-type plasminogen activator	t-PA	P00750
Transferrin receptor protein 1	TR	P02786
Trefoil factor 3	TFF3	Q07654
Trem-like transcript 2 protein	TLT-2	Q5T2D2
Tumor necrosis factor ligand superfamily member 13B	TNFSF13B	Q9Y275

Tumor necrosis factor receptor 1	TNF-R1	P19438
Tumor necrosis factor receptor 2	TNF-R2	P20333
Tumor necrosis factor receptor superfamily member 10C	TNFRSF10C	O14798
Tumor necrosis factor receptor superfamily member 14	TNFRSF14	Q92956
Tumor necrosis factor receptor superfamily member 6	FAS	P25445
Tyrosine-protein kinase receptor UFO	AXL	P30530
Tyrosine-protein phosphatase non-receptor type substrate 1	SHPS-1	P78324
Urokinase plasminogen activator surface receptor	U-PAR	Q03405
Urokinase-type plasminogen activator	uPA	P00749
von Willebrand factor	vWF	P04275

2.5. Clinical outcome assessment

The clinical outcome of interest in this study was unexpected hospitalization for heart failure that necessitated intravenous diuretics and all-cause mortality. These outcomes were assessed in the imaging cohort by review of medical records, reports from family members, and official mortality data from Statistics Korea. Patients were followed from the date of CMR to the last clinical follow-up or death.

2.6. Statistical analysis

Continuous data are presented as mean±standard deviation or median (interquartile range) depending on the normality of distribution, and categorical data as number (%). Characteristics were compared between the groups using the t-test (or Mann-Whitney test for non-normally distributed continuous variables) or the chi-square test, as appropriate. Comparisons of groups according to diabetes medication was conducted using the Kruskal-Wallis test. Variables associated with increased diffuse interstitial or replacement fibrosis were analyzed using logistic regression and the degree of association expressed in odds ratio (OR) with 95% confidence interval (CI). Multivariable models were constructed with the stepwise backward selection method using the Akaike information criterion or inclusion of clinically important variables such as age, sex, diabetes, hypertension, atrial fibrillation, IHD, and peak aortic velocity.

Comparison of plasma biomarker levels according to the diabetic status was performed using the Welch's two-sample t-test, adjusting for multiple testing with the Benjamini & Hochberg method. The adjusted p-values represent the false discovery rate and p-values <0.05 were considered significant. Logistic regression was used to assess the association of plasma biomarkers with diabetic status, adjusting for age, sex, hypertension, atrial fibrillation, IHD, and peak aortic velocity. Functional enrichment analyses were performed using g:Profiler with Gene Ontology terms. Kaplan-Meier survival curves with log-rank tests were used to compare event-free survival according to the presence of diabetes. Cox proportional-hazards regression analyses were used to assess predictors of the endpoints and the effect size expressed as hazard ratio (HR) with 95% CI. The final multivariable model was constructed with stepwise backward selection from clinically important variables such as age, sex, diabetes, hypertension, atrial fibrillation, stroke, IHD, peak aortic velocity, LV ejection fraction by echocardiography, and AVR. Two-sided p-values <0.05 were considered statistically significant. Analyses were conducted using R version 4.0 (Vienna, Austria) or SPSS version 25 (Chicago, USA).

Chapter 3. Results

3.1. Demographic and clinical characteristics according to the presence of diabetes

In the imaging cohort (n=253), there were 66 patients with diabetes (26.1%). Among the diabetic patients, 48 (72.7%) were on oral medication only, 5 (7.6%) on insulin, and 13 (19.7%) on no medication. The diabetic patients were older (70.4±6.8 vs. 66.7±10.1 years, p=0.001), had a higher prevalence of hypertension (72.7% vs. 55.1%, p=0.018), IHD (28.8% vs. 9.1%, p<0.001), and tended to use more diuretics compared to non-diabetic patients (Table 3).

In the biomarker cohort (n=100), there were 27 patients with diabetes (27%), of whom 18 (66.7%) were on oral medication only, 6 (22.2%) on insulin, and 3 (11.1%) on no medication (Table 3). Because the size of the biomarker cohort was smaller than that of the imaging cohort, there were no statistical difference in the clinical or demographic parameters between diabetic and non-diabetic patients in the biomarker cohort.

3.2. Increased risk of myocardial fibrosis on noninvasive imaging in diabetic AS patients

In the imaging cohort, the diabetic patients compared to the non-diabetic patients had worse LV diastolic function (prevalence of LV diastolic dysfunction 79.7% vs. 53.5%, p=0.001), supported by

Table 3. Demographic and clinical characteristics of the patients in the imaging cohort and biomarker cohort.

	Imaging cohort				Biomarker cohort			
	Total (n=253)	Non-DM (N=187)	DM (N=66)	p-value	Total (n=100)	Non-DM (N=73)	DM (N=27)	p-value
Age (years)	67.7±9.5	66.7±10.1	70.4±6.8	0.001	66.6±9.6	65.5±9.9	69.5±8.2	0.064
Male	127 (50.2)	93 (49.7)	34 (51.5)	0.916	60 (60.0)	43 (58.9)	17 (63.0)	0.890
Body surface area (m^2)	1.65±0.16	1.65±0.16	1.67±0.14	0.381	1.70±0.16	1.69±0.16	1.73±0.14	0.275
Hypertension	151 (59.7)	103 (55.1)	48 (72.7)	0.018	58 (58.0)	39 (53.4)	19 (70.4)	0.195
Atrial fibrillation	31 (12.3)	18 (9.6)	13 (19.7)	0.054	13 (13.0)	11 (15.1)	2 (7.4)	0.499
Stroke	21 (8.3)	13 (7.0)	8 (12.1)	0.294	11 (11.0)	6 (8.2)	5 (18.5)	0.271
Ischemic heart disease	36 (14.2)	17 (9.1)	19 (28.8)	<0.001	10 (10.0)	5 (6.8)	5 (18.5)	0.177
Creatinine (mg/dL)	0.91±0.53	0.86±0.20	1.03±0.98	0.180	1.12±1.37	1.07±1.19	1.25±1.80	0.635
Euroscore II	1.6±1.5	1.3±0.7	2.5±2.5	<0.001	1.7±1.8	1.7±1.5	1.8±2.3	0.717
NYHA III-IV	55 (21.8)	36 (19.4)	19 (28.8)	0.155	16 (16.0)	13 (17.8)	3 (11.1)	0.614

Medication								
ACE inhibitor/ARB	111 (43.9)	75 (40.1)	36 (54.5)	0.059	37 (37.0)	25 (34.2)	12 (44.4)	0.481
Beta-blocker	116 (45.8)	88 (47.1)	28 (42.4)	0.613	48 (48.0)	33 (45.2)	15 (55.6)	0.487
Calcium channel blocker	56 (22.1)	43 (23.0)	13 (19.7)	0.702	-	-	-	
Diuretics	99 (39.1)	64 (34.2)	35 (53.0)	0.011	-	-	-	
Diabetes medication								
None			13 (19.7)				3 (11.1)	
Oral medication only			48 (72.7)				18 (66.7)	
Insulin user			5 (7.6)				6 (22.2)	

ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; DM, diabetes mellitus; NYHA, New York Heart Association.

lower e′ velocity, higher E/e′ and tricuspid regurgitation peak velocity, and a shorter mitral deceleration time (Table 4). Notably, the prevalence of LV diastolic dysfunction was higher in the diabetic patients (Figure 2A). When stratified by diabetes medication as a surrogate marker of chronicity and severity of diabetes, there was also a higher prevalence of LV diastolic dysfunction with need for more intensive diabetes treatment (Figure 2B, p=0.003). However, the peak aortic velocity was lower in patients with diabetes (4.5±0.9 vs. 4.8±8.0 m/s, p=0.036). In the biomarker cohort, the diabetic patients had lower e′ velocity with a tendency towards more advanced LV diastolic dysfunction than the nondiabetic patients. Otherwise, there were no significant differences in other echocardiography indices (Table 5).

Table 4. Echocardiography and cardiac magnetic resonance analysis of the patients with and without diabetes in the imaging cohort.

	Total (n=253)	Non-DM (N=187)	DM (N=66)	p-value
Echocardiography				
LVEDD (mm)	50.2±6.7	50.1±6.8	50.6±6.4	0.633
LVESD (mm)	31.8±7.8	31.5±7.4	32.4±9.0	0.423
LV mass index (g/m^2)	132±40	133±41	130±37	0.625
Relative wall thickness	0.44±0.09	0.44±0.09	0.44±0.09	0.974
LV ejection fraction (%)	59.6±9.9	60.3±8.9	57.6±12.0	0.104
LA diameter (mm)	43.8±6.9	43.4±7.1	44.8±6.2	0.181
E velocity (m/s)	0.79±0.38	0.76±0.36	0.88±0.43	0.038

A velocity (m/s)	0.87±0.29	0.86±0.30	0.90±0.26	0.335
Deceleration time (ms)	247±79	253±83	229±64	0.022
E/A	0.96±0.58	0.95±0.58	0.96±0.61	0.894
e′ velocity (cm/s)	4.6±1.4	4.7±1.4	4.2±1.4	0.007
a′ velocity (cm/s)	7.3±1.8	7.3±1.7	7.2±2.1	0.714
s′ velocity (cm/s)	5.1±1.4	5.2±1.4	4.9±1.5	0.132
E/e′	18.6±10.4	16.9±8.1	23.4±14.0	0.001
TR Vmax (m/s)	2.5±0.4	2.5±0.4	2.7±0.6	0.013
PASP (mmHg)	34.4±9.4	33.3±8.0	38.0±12.4	0.015
LAVI (mL/m^2)	52.8±18.7	53.5±20.0	50.5±13.6	0.170
Peak AV velocity (m/s)	4.7±0.8	4.8±0.8	4.5±0.9	0.036
AV mean PG (mmHg)	55±21	56±22	51±21	0.095
AV area (cm^2)	0.76±0.23	0.76±0.22	0.74±0.25	0.371
Presence of LVDD (n=231)	139 (60.2)	92 (53.5)	47 (79.7)	0.001
LVDD grade (n=208)				0.011
Normal	40 (19.2)	38 (23.9)	2 (4.1)	
Indeterminate	52 (25.0)	42 (26.4)	10 (20.4)	
Grade 1 LVDD	22 (10.6)	14 (8.8)	8 (16.3)	
Grade 2 LVDD	84 (40.4)	58 (36.5)	26 (53.1)	
Grade 3 LVDD	10 (4.8)	7 (4.4)	3 (6.1)	

AV, aortic valve; DM, diabetes mellitus; LA, left atrial; LAVI, LA volume index; LV, left ventricular; LVDD, LV diastolic dysfunction; LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension; PASP, pulmonary artery systolic pressure; PG, pressure gradient; TR, tricuspid regurgitation; Vmax, maximal velocity.

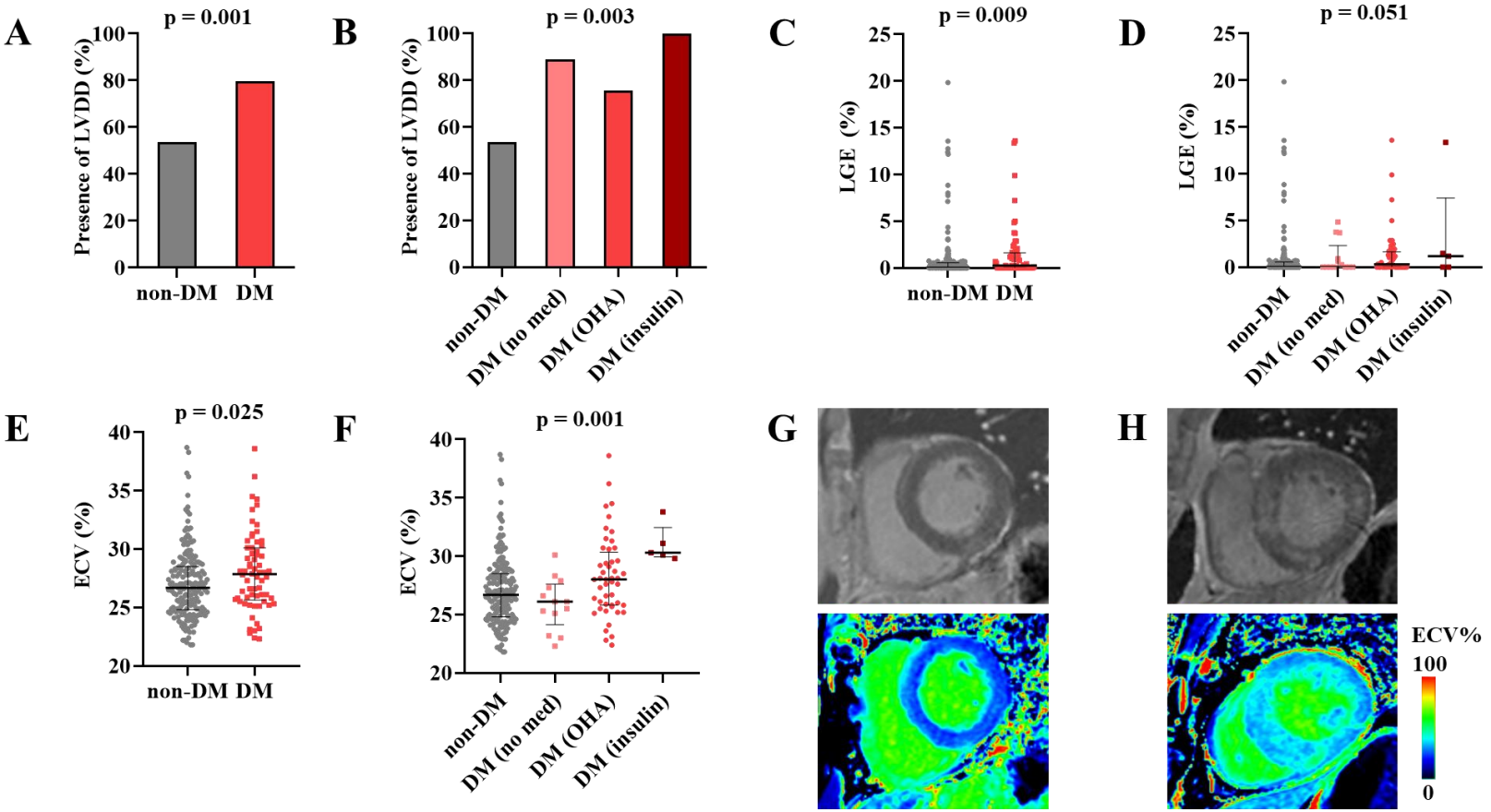


Figure 2. Comparison of myocardial fibrosis and left ventricular diastolic function in AS patients according to diabetes

and diabetes medication status.

(A, B) Comparison of the degree of diastolic dysfunction in (A) patients with versus without diabetes and in (B) patients stratified by the diabetes medication status. (C, D) Comparison of late gadolinium enhancement (LGE) in (C) patients with versus without diabetes and in (D) patients stratified by the diabetes medication status. (E, F) Comparison of extracellular volume (ECV) in (E) patients with versus without diabetes and in (F) patients stratified by the diabetes medication status. (G, H) Representative LGE and ECV images of CMR taken from (G) a non-diabetic AS patient (no LGE; ECV 26.4%) versus (H) a diabetic AS patient on OHA treatment (midwall LGE in the septal and lateral wall; ECV 34.5%).

AS, aortic stenosis; CMR, cardiovascular magnetic resonance; DM, diabetes mellitus; ECV, extracellular volume fraction; LGE, late gadolinium enhancement; OHA, oral hypoglycemic agents.

Table 5. Echocardiography analysis of the patients with and without diabetes in the imaging cohort.

	Total (n=100)	Non-DM (N=73)	DM (N=27)	p-value
Echocardiography				
LVEDD (mm)	48.8±6.9	49.0±7.3	48.3±6.0	0.666
LVESD (mm)	31.3±7.0	31.4±6.9	31.2±7.4	0.907
LV mass index (g/m^2)	128±55	130±59	124±42	0.599
Relative wall thickness	0.46±0.09	0.46±0.09	0.47±0.07	0.683
LV ejection fraction (%)	59.9±9.2	60.6±9.1	57.9±9.5	0.203
LA diameter (mm)	43.8±8.5	43.5±8.9	44.6±7.4	0.561
E velocity (m/s)	0.74±0.35	0.77±0.37	0.65±0.27	0.168
A velocity (m/s)	0.85±0.25	0.82±0.26	0.93±0.18	0.054
Deceleration time (ms)	243±96	248±102	230±80	0.431
E/A	0.86±0.42	0.92±0.46	0.71±0.24	0.894
e′ velocity (cm/s)	4.6±1.5	4.9±1.6	4.0±1.1	0.016
a′ velocity (cm/s)	7.1±1.9	7.1±1.9	7.2±1.8	0.959
s′ velocity (cm/s)	4.9±1.4	4.9±1.4	4.8±1.4	0.911
E/e′	16.9±8.6	16.9±9.6	16.9±5.2	0.943
TR Vmax (m/s)	2.5±0.5	2.5±0.5	2.5±0.5	0.668
PASP (mmHg)	36.2±11.2	36.6±11.5	35.0±10.6	0.673
LAVI (mL/m^2)	44.5±19.3	44.2±17.0	45.4±24.9	0.819
Peak AV velocity (m/s)	4.6±0.8	4.6±0.8	4.6±0.7	0.781
AV mean PG (mmHg)	53±18	52±18	54±17	0.992
AV area (cm^2)	0.78±0.26	0.79±0.27	0.74±0.20	0.826

Presence of LVDD (n=87)	41 (47.1)	26 (41.9)	15 (60.0)	0.197
LVDD grade (n=77)				0.309
Normal	28 (36.4)	22 (40.0)	6 (27.3)	
Indeterminate	18 (23.4)	14 (25.5)	4 (18.2)	
Grade 1 LVDD	4 (5.2)	3 (5.5)	1 (4.5)	
Grade 2 LVDD	25 (32.5)	14 (25.5)	11 (50.0)	
Grade 3 LVDD	2 (2.6)	2 (3.6)	0 (0)	

AV, aortic valve; DM, diabetes mellitus; LA, left atrial; LAVI, LA volume index; LV, left ventricular; LVDD, LV diastolic dysfunction; LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension; PASP, pulmonary artery systolic pressure; PG, pressure gradient; TR, tricuspid regurgitation; Vmax, maximal velocity.

On analysis of CMR in the participants of the imaging cohort, a significant increase of replacement fibrosis was observed in diabetic patients (**Table 6**). The LGE was present in 56% of diabetic patients compared to 40% of non-diabetic patients (**Figure 3**, p=0.036). The extent of LGE was also higher in the diabetic patients (**Figure 2C**, LGE% in the entire population 0.3 [0.0-1.6] vs. 0.0 [0.0-0.5], p=0.009) (LGE% in those with any LGE 1.2 [0.4-2.9] vs. 0.6 [0.2-1.5], p=0.026). There was also a tendency for higher LGE% with more intensive diabetes treatment (p=0.051) (**Figure 2D**, **Table 7**).

As for the degree of diffuse interstitial fibrosis, the ECV was higher in patients with diabetes (**Figure 2E**, ECV% 27.9 [25.7-30.1] vs. 26.7 [24.9-28.5], p=0.025). Similar to the analysis of LGE%, the ECV% was significantly higher with more intensive treatment of diabetes (**Figure 2F**, p=0.001) (**Table 7**). The LV stroke volume and ejection fraction measured by CMR were lower in diabetic patients.

Table 6. Cardiac magnetic resonance analysis of the patients with and without diabetes in the imaging cohort.

	Total (n=253)	Non-DM (N=187)	DM (N=66)	p-value
Cardiac magnetic resonance				
Indexed LVEDV (mL/m^2)	109.3±47.8	112.2±50.8	100.9±36.8	0.056
Indexed LVESV (mL/m^2)	45.1±34.3	45.6±35.9	43.8±29.3	0.715

Indexed LVSV (mL/m^2)	58.0±17.4	60.5±18.0	50.9±13.2	<0.001
LV ejection fraction (%)	62.9±13.8	64.0±13.5	59.5±14.0	0.021
LV mass index (g/m^2)	101.6±36.7	101.6±37.0	101.3±36.1	0.950
LV mass/volume ratio* (g/mL)	1.00±0.34	0.98±0.35	1.05±0.32	0.191
ECV (%)	26.8 [25.1−28.9]	26.7 [24.9−28.5]	27.9 [25.7−30.1]	0.025
Presence of LGE	112 (44.3)	75 (40.1)	37 (56.1)	0.036
LGE (%)	0.0 [0.0−0.7]	0.0 [0.0−0.5]	0.3 [0.0−1.6]	0.009
LGE (%) in patients with LGE	0.8 [0.2−1.9]	0.6 [0.2−1.5]	1.2 [0.4−2.9]	0.026

*LV mass divided by LV end−diastolic volume.

ECV, extracellular volume fraction; DM, diabetes mellitus; LGE, late gadolinium enhancement; LV, left ventricular; LVEDV, LV end−diastolic volume; LVESV, LV end−systolic volume; LVSV, LV stroke volume.

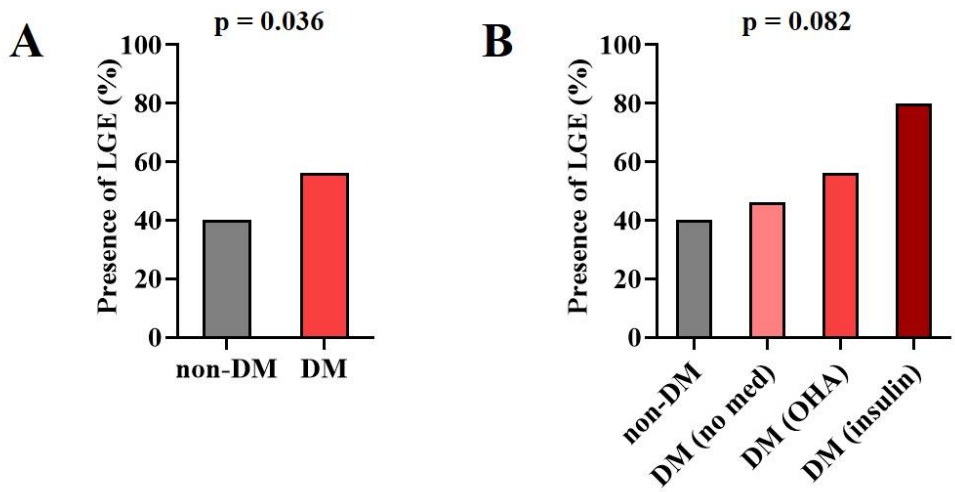


Figure 3. Comparison of presence of LGE in AS patients according to diabetes and diabetes medication status.

Comparison of the presence of late gadolinium enhancement (LGE) in (A) patients with versus without diabetes and in (B) patients stratified by the diabetes medication status.

AS, aortic stenosis; DM, diabetes mellitus; LGE, late gadolinium enhancement.

Table 7. Comparison of myocardial fibrosis and left ventricular diastolic function according to diabetes medication in AS patients.

	Non-DM (n=187)	DM (no medication) (n=13)	DM (OHA only) (n=48)	DM (insulin) (n=5)	p-value
Presence of LVDD	92 (53.5)	8 (88.9)	34 (75.6)	5 (100.0)	0.003
Presence of LGE	75 (40.1)	6 (46.2)	27 (56.2)	4 (80.0)	0.082
LGE (%)	0 [0-0.5]	0 [0-0.9]	0.3 [0-1.6]	1.2 [0-1.5]	0.051
ECV (%)	26.7 [24.9-28.5]	26.1 [25.1-27.3]	28.0 [25.8-30.1]	30.3 [30.1-31.1]	0.002

DM, diabetes mellitus; ECV, extracellular volume fraction; LGE, late gadolinium enhancement; LVDD, left ventricular diastolic dysfunction; OHA, oral hypoglycemic agents.

Diabetes was significantly associated with increased diffuse interstitial and replacement fibrosis on univariable and multivariable analyses (Table 8). The presence of diabetes was associated with the highest quartile of both ECV% (HR 2.08, 95% CI 1.06−4.06, p=0.033) and LGE% (HR 2.82, 95% CI 1.45−5.50, p=0.002), after adjustment for age, sex, hypertension, atrial fibrillation, IHD, and peak aortic velocity.

Table 8. Predictors of increased diffuse interstitial or replacement myocardial fibrosis (highest quartile).

Diffuse myocardial fibrosis (ECV)						
	Univariable		Multivariable Model 1*		Multivariable Model 2†	
	Crude HR	p-value	Adjusted HR	p-value	Adjusted HR	p-value
Age (years)	1.00 (0.97-1.03)	0.772			0.98 (0.95-1.02)	0.280
Male	1.18 (0.66-2.11)	0.578			1.19 (0.66-2.16)	0.566
Diabetes	2.17 (1.17-4.04)	0.015	2.17 (1.17-4.04)	0.015	2.08 (1.06-4.06)	0.033
Hypertension	1.48 (0.80-2.71)	0.208			1.39 (0.72-2.68)	0.326
Atrial fibrillation	0.93 (0.38-2.28)	0.874			0.84 (0.33-2.12)	0.707
Ischemic heart disease	1.51 (0.69-3.28)	0.300			1.16 (0.50-2.66)	0.732
Peak aortic velocity (m/s)	0.76 (0.54-1.09)	0.139			0.81 (0.56-1.17)	0.269
Replacement fibrosis (LGE)						
	Univariable		Multivariable Model 1*		Multivariable Model 2†	

	Crude HR	p-value	Adjusted HR	p-value	Adjusted HR	p-value
Age (years)	1.03 (0.99-1.06)	0.100			1.01 (0.98-1.05)	0.452
Male	2.17 (1.20-3.93)	0.010	2.13 (1.14-3.97)	0.017	2.17 (1.16-4.07)	0.016
Diabetes	3.32 (1.80-6.12)	<0.001	2.92 (1.53-5.57)	0.001	2.82 (1.45-5.50)	0.002
Hypertension	1.44 (0.79-2.62)	0.235			1.25 (0.64-2.43)	0.514
Atrial fibrillation	1.30 (0.57-3.01)	0.533			1.06 (0.43-2.64)	0.894
Ischemic heart disease	3.42 (1.64-7.11)	0.001	2.37 (1.09-5.15)	0.029	2.38 (1.08-5.20)	0.031
Peak aortic velocity (m/s)	1.03 (0.73-1.45)	0.869			1.22 (0.84-1.78)	0.294

Important clinical variables are shown in the first column. *Model 1 was constructed with stepwise backward selection from variables presented in the first column. †Model 2 was adjusted for all variables in the first column.

ECV, extracellular volume fraction; HR, hazard ratio; LGE, late gadolinium enhancement.

3.3. Upregulation of the proinflammatory and profibrotic pathways in the plasma proteome of diabetic AS patients

The distribution of NPX values for each sample are shown in Figure 4. Among the 92 candidate proteins in the plasma proteomics analysis of the biomarker cohort, 9 proteins (E-selectin, interleukin-1 receptor type 1, interleukin-1 receptor type 2, galectin-4, intercellular adhesion molecule 2, integrin beta-2, galectin-3, growth differentiation factor 15 [GDF-15], and cathepsin D) were significantly upregulated in diabetic AS patients compared to non-diabetic AS patients (false discovery rate <5%) (Figure 5, Table 9). There were no proteins that were significantly downregulated in diabetic AS patients.

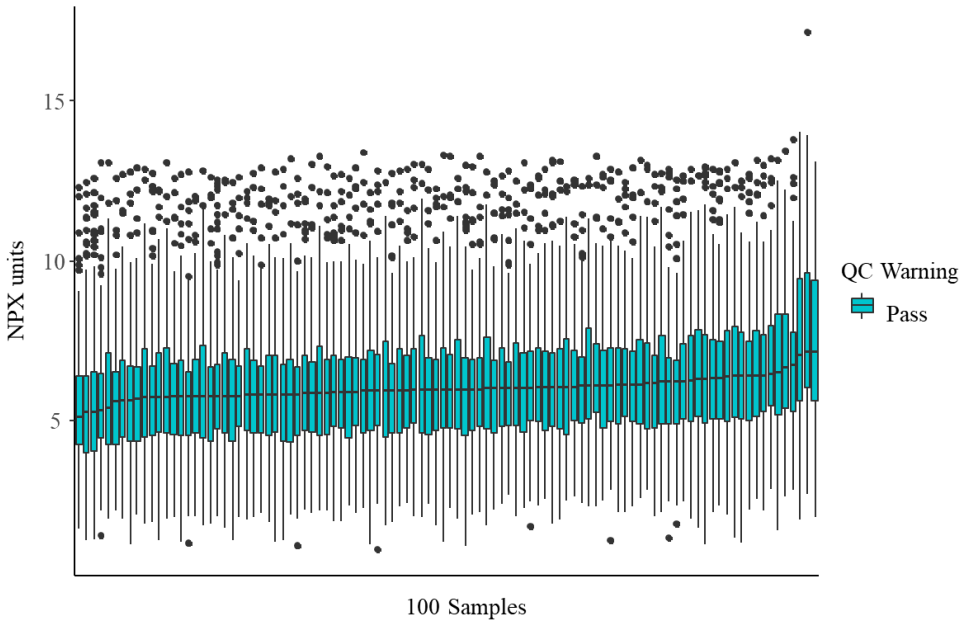


Figure 4. Distribution of NPX values for each sample.

All samples passed quality control and protein levels were normalized and presented in NPX (normalized protein expression) units using the Log_2 scale (1 NPX difference equaling 2−fold change in protein concentration).

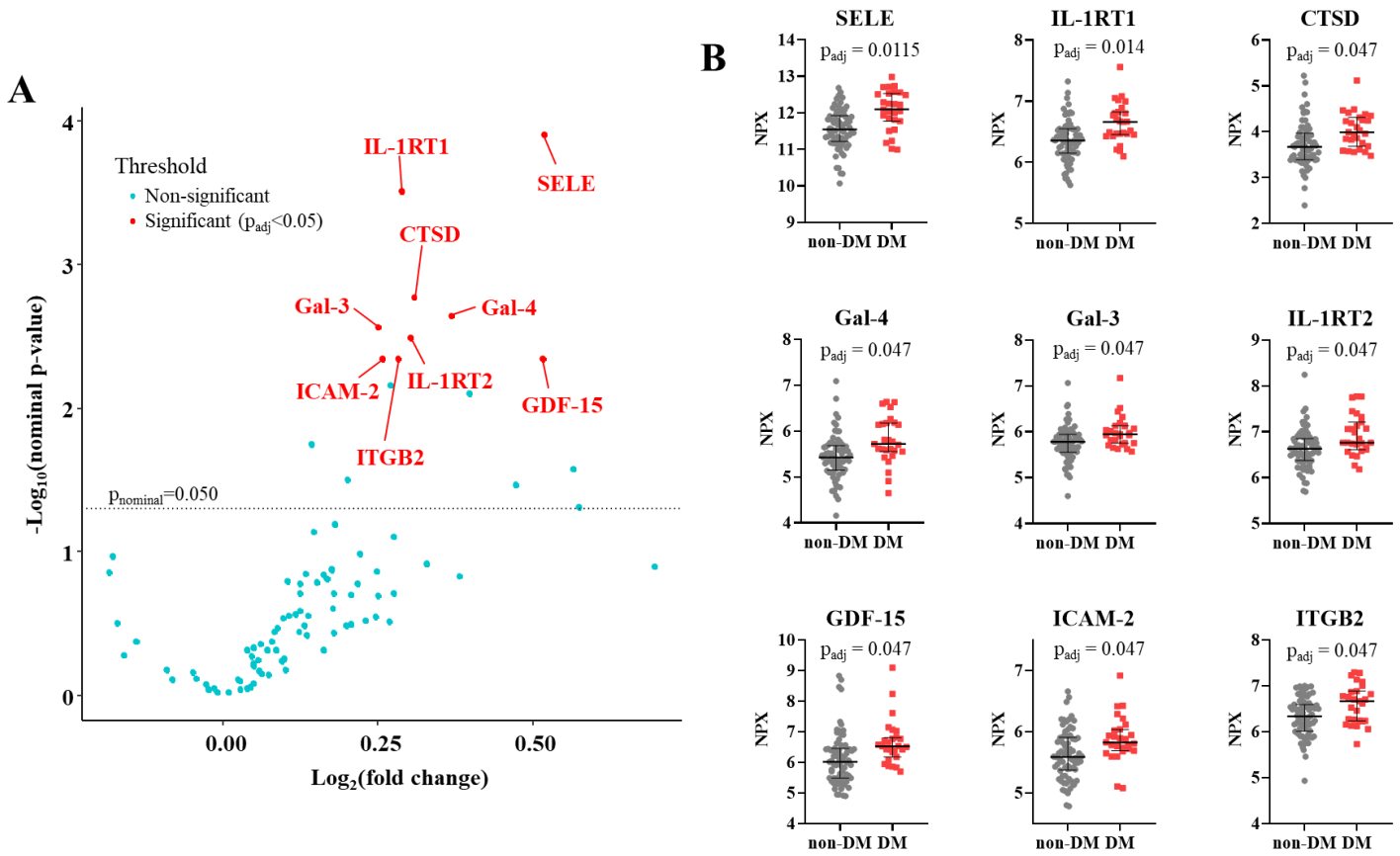


Figure 5. Significantly upregulated plasma proteins in AS patients with diabetes by proteomic analysis.

(**A**) Volcano plot identifying the significantly increased plasma proteins in diabetic AS patients (annotated in red). (**B**) Scatter plots comparing the plasma levels of differentially expressed proteins in AS patients with and without diabetes. All comparisons were adjusted for multiple testing with the Benjamini & Hochberg method, and the adjusted p-values represent the false discovery rate. The horizontal bars for each groups indicate median and interquartile range.

adj, adjusted; AS, aortic stenosis; SELE, E-selectin; IL-1RT1, interleukin-1 receptor type 1; CTSD, cathepsin D; Gal-4, galectin-4; Gal-3, galectin-3; IL-1RT2, interleukin-1 receptor type 2; GDF-15, growth differentiation factor 15; ICAM-2, intercellular adhesion molecule 2; ITGB2, integrin beta-2.

Table 9. Comparison of plasma biomarker expression in AS patients with and without diabetes.

Biomarker	Diabetes* (n=27)	Non-diabetic* (n=73)	Difference (log_2 scale)*	Nominal p-value	Adjusted p-value†
SELE	12.069	11.552	0.518	0.0001	0.0115
IL-1RT1	6.647	6.359	0.289	0.0003	0.0141
CTSD	4.03	3.721	0.309	0.0017	0.0468
Gal-4	5.805	5.436	0.369	0.0023	0.0468
Gal-3	5.995	5.745	0.25	0.0027	0.0468
IL-1RT2	6.931	6.629	0.303	0.0033	0.0468
GDF-15	6.648	6.132	0.516	0.0045	0.0468
ICAM-2	5.883	5.625	0.257	0.0045	0.0468
ITGB2	6.604	6.321	0.283	0.0046	0.0468
IL-18BP	7.095	6.825	0.27	0.0069	0.0638
CCL16	8.005	7.607	0.398	0.0079	0.0661
ALCAM	7.77	7.627	0.143	0.0177	0.1357
t-PA	6.04	5.475	0.565	0.0266	0.1885
CD163	9.268	9.067	0.201	0.0314	0.2064
PSP-D	3.795	3.323	0.472	0.0345	0.2117
IGFBP-1	7.278	6.703	0.575	0.0488	0.2804
TR-AP	5.964	5.783	0.181	0.0649	0.3514
GRN	6.098	5.953	0.145	0.0723	0.3695
U-PAR	6.394	6.119	0.275	0.0790	0.3826
AP-N	6.508	6.288	0.22	0.1040	0.4411

COL1A1	2.632	2.811	−0.179	0.1069	0.4411
TFF3	5.687	5.36	0.327	0.1225	0.4411
CHIT1	5.106	4.41	0.696	0.1264	0.4411
IL2−RA	3.859	3.684	0.175	0.1319	0.4411
uPA	6.079	5.905	0.174	0.1333	0.4411
TNF−R2	6.724	6.475	0.248	0.1354	0.4411
PON3	6.536	6.721	−0.185	0.1396	0.4411
AXL	9.72	9.587	0.133	0.1412	0.4411
EPHB4	6.55	6.387	0.163	0.1446	0.4411
FABP4	6.011	5.63	0.382	0.1494	0.4411
MCP−1	5.115	4.948	0.167	0.1546	0.4411
IL−6RA	12.831	12.728	0.103	0.1593	0.4411
TLT−2	6.165	6.014	0.15	0.1647	0.4411
CTSZ	5.115	4.991	0.123	0.1664	0.4411
CPA1	6.46	6.243	0.217	0.1678	0.4411
SELP	9.969	9.79	0.178	0.1940	0.4665
TNFRSF10C	6.378	6.255	0.123	0.1945	0.4665
PI3	3.312	3.038	0.275	0.1968	0.4665
FAS	7.053	6.848	0.206	0.1977	0.4665
TNFRSF14	5.028	4.779	0.249	0.2046	0.4705
CPB1	6.579	6.403	0.176	0.2475	0.5553
OPG	4.411	4.286	0.124	0.2556	0.5599
SHPS−1	4.255	4.139	0.116	0.2720	0.5638
PECAM−1	5.306	5.169	0.137	0.2787	0.5638
TNFSF13B	7.763	7.657	0.105	0.2810	0.5638

CSTB	4.881	4.635	0.245	0.2819	0.5638
CDH5	4.997	4.901	0.096	0.2906	0.5688
SCGB3A2	3.358	3.129	0.229	0.3004	0.5719
PAI	6.358	6.09	0.268	0.3089	0.5719
Ep-CAM	5.812	5.984	-0.171	0.3147	0.5719
RETN	7.058	6.852	0.206	0.3208	0.5719
TNF-R1	6.965	6.767	0.199	0.3267	0.5719
GP6	2.803	2.672	0.131	0.3295	0.5719
Notch-3	6.188	6.102	0.086	0.3395	0.5785
CNTN1	4.364	4.282	0.082	0.3617	0.5963
TR	6.576	6.454	0.121	0.3636	0.5963
PDGF-subunit-A	3.012	2.832	0.179	0.3694	0.5963
LTBR	4.027	3.892	0.135	0.3789	0.6010
CD93	10.558	10.479	0.079	0.4182	0.6455
MEPE	5.344	5.484	-0.14	0.4210	0.6455
RARRES2	12.194	12.133	0.06	0.4399	0.6635
CXCL16	6.292	6.244	0.049	0.4663	0.6784
JAM-A	6.349	6.187	0.162	0.4809	0.6784
IGFBP-7	11.335	11.265	0.07	0.4833	0.6784
EGFR	3.092	3.055	0.037	0.4852	0.6784
TIMP4	4.392	4.308	0.084	0.4867	0.6784
MMP-3	7.231	7.393	-0.162	0.5211	0.7155
IL-17RA	5.027	4.983	0.045	0.5364	0.7257
PRTN3	5.176	5.078	0.098	0.5555	0.7407

PLC	8.668	8.611	0.056	0.5734	0.7500
MMP-9	5.842	5.748	0.094	0.5788	0.7500
MMP-2	5.294	5.245	0.048	0.6039	0.7717
MPO	3.801	3.751	0.049	0.6266	0.7897
ST2	5.66	5.561	0.099	0.6616	0.8121
IGFBP-2	7.681	7.774	-0.093	0.6620	0.8121
PGLYRP1	8.229	8.171	0.058	0.6756	0.8178
CCL24	6.313	6.363	-0.05	0.6926	0.8275
BLM-hydrolase	2.81	2.748	0.062	0.7119	0.8397
CCL15	8.514	8.441	0.073	0.7263	0.8458
OPN	9.221	9.265	-0.044	0.7712	0.8766
PCSK9	3.428	3.404	0.024	0.7738	0.8766
NT-proBNP	9.143	9.227	-0.083	0.7813	0.8766
KLK6	4.659	4.631	0.028	0.7979	0.8844
DLK-1	6.401	6.352	0.049	0.8204	0.8986
LDL-receptor	5.105	5.133	-0.027	0.8378	0.9068
CASP-3	6.525	6.482	0.043	0.8781	0.9329
AZU1	4.068	4.031	0.037	0.8953	0.9329
TFPI	9.751	9.766	-0.015	0.8960	0.9329
MB	8.87	8.894	-0.024	0.9071	0.9329
CHI3L1	7.372	7.346	0.026	0.9126	0.9329
vWF	10.031	10.041	-0.009	0.9569	0.9571
SPON1	2.839	2.831	0.008	0.9571	0.9571

*NPX units (log_2 scale): relative quantification unit logarithmically related

to protein concentration.

†False discovery rate by adjustment with the Benjamini–Hochberg method.

Abbreviations for the name of each proteins: refer to Table 2.

These proteins biomarkers were independently associated with diabetic status after adjustment for age, sex, atrial fibrillation, IHD, peak aortic velocity, and LV ejection fraction, with odds ratios ranging from 2.97 to 14.2 per 2-fold increase in protein level (Table 10). Pathway over-representation analyses of the plasma proteome indicated that pathways related to proinflammatory response and extracellular matrix components were enriched in the plasma of AS patients with concomitant diabetes (Table 11, Figure 6).

Table 10. Independent association of differentially regulated plasma biomarkers with presence of diabetes.

Biomarker	Mean level					Association with diabetic status‡			
	DM* (n=27)	Non-DM* (n=73)	Difference (log_2 scale)*	Nominal p-value	Adj. p-value†	Unadj. OR (95% CI)	p-value	Adj. OR§ (95% CI)	p-value
E-selectin	12.069	11.552	0.518	0.0001	0.0115	6.62 (2.57-19.9)	<0.001	6.99 (2.49-23.6)	<0.001
Interleukin-1 receptor type 1	6.647	6.359	0.289	0.0003	0.0141	12.7 (3.09-65.6)	0.001	14.2 (2.65-104)	0.004
Cathepsin D	4.03	3.721	0.309	0.0017	0.0468	3.98 (1.53-11.5)	0.007	4.84 (1.64-15.7)	0.006
Galectin-4	5.805	5.436	0.369	0.0023	0.0468	4.42 (1.75-12.6)	0.003	4.85 (1.65-16.4)	0.006
Galectin-3	5.995	5.745	0.25	0.0027	0.0468	6.33 (1.79-27.7)	0.007	8.12 (1.83-47.0)	0.011
Interleukin-1	6.931	6.629	0.303	0.0033	0.0468	5.05	0.005	7.91	0.005

receptor type 2						(1.75−16.8)		(2.03−37.8)	
Growth differentiation factor 15	6.648	6.132	0.516	0.0045	0.0468	2.02 (1.20−3.61)	0.011	2.97 (1.28−8.02)	0.018
Intercellular adhesion molecule 2	5.883	5.625	0.257	0.0045	0.0468	5.02 (1.61−17.6)	0.008	4.21 (1.20−17.1)	0.031
Integrin beta−2	6.604	6.321	0.283	0.0046	0.0468	5.62 (1.78−20.4)	0.005	8.36 (2.24−39.2)	0.003

*NPX units (log_2 scale): relative quantification unit logarithmically related to protein concentration.

†False discovery rate by adjustment with the Benjamini−Hochberg method (<5% considered significant)

‡Per 2−fold increase in protein level.

§Logistic regression adjusted for age, sex, atrial fibrillation, ischemic heart disease, peak aortic velocity, and left ventricular ejection fraction.

Adj., adjusted; DM, diabetes mellitus.

Table 11. Functional enrichment analysis of the plasma proteome according to the presence of diabetes in patients with aortic stenosis.

GO domain	Over-represented pathways (GO terms)	GO term ID	Adjusted p-value	Term size	Query size	Intersection size	Intersections (UniProt IDs)
MF	Interleukin-1 receptor activity	GO:0004908	0.00029	7	6	2	P14778, P27930
MF	Interleukin-1 binding	GO:0019966	0.00049	9	6	2	P14778, P27930
MF	Carbohydrate binding	GO:0030246	0.00503	277	5	3	P16581, P56470, P17931
MF	Interleukin-1, type I, activating receptor activity	GO:0004909	0.03410	2	2	1	P14778
BP	Regulation of cellular extravasation	GO:0002691	0.00412	33	2	2	P16581, P14778
BP	Regulation of interleukin-1-mediated signaling pathway	GO:2000659	0.00526	10	6	2	P14778, P27930
BP	Cellular extravasation	GO:0045123	0.00560	69	9	3	P16581, P14778,

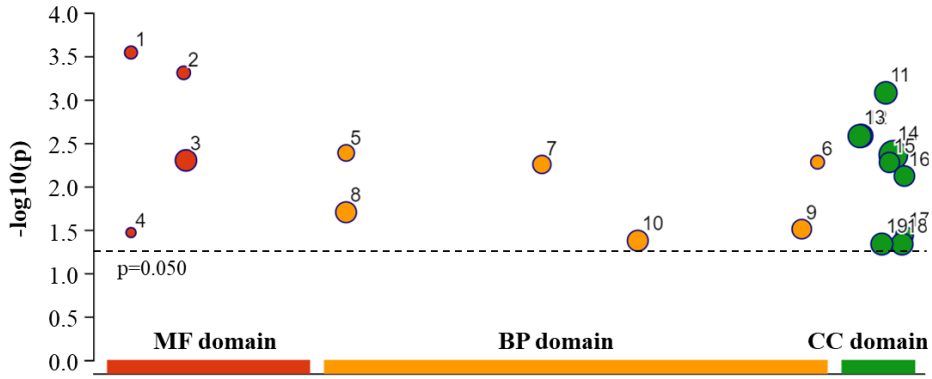
							P05107
BP	Regulation of leukocyte migration	GO:0002685	0.01989	212	5	3	P16581, P14778, P17931
BP	Neutrophil migration	GO:1990266	0.03112	122	9	3	P14778, P17931, P05107
BP	Response to interleukin-1	GO:0070555	0.04227	217	6	3	P16581, P14778, P27930
CC	Collagen-containing extracellular matrix	GO:0062023	0.00084	421	7	4	P07339, P56470, P17931, Q99988
CC	Extracellular matrix	GO:0031012	0.00262	562	7	4	P07339, P56470, P17931, Q99988
CC	External encapsulating structure	GO:0030312	0.00264	563	7	4	P07339, P56470, P17931, Q99988
CC	Cell periphery	GO:0071944	0.00433	6178	9	9	P16581, P14778,

							P07339, P56470, P17931, P27930, Q99988, P13598, P05107
CC	Tertiary granule*	GO:0070820	0.00531	163	9	3	P07339, P17931, P05107
CC	Ficolin-1-rich granule†	GO:0101002	0.00761	184	9	3	P07339, P17931, P05107
CC	Ficolin-1-rich granule membrane	GO:0101003	0.03677	60	9	2	P17931, P05107
CC	Membrane microdomain	GO:0098857	0.04622	339	9	3	P16581, P07339, P05107
CC	Membrane raft	GO:0045121	0.04622	339	9	3	P16581, P07339, P05107

*Secretory granule containing cathepsin and gelatinase found primarily in mature neutrophil cells; readily exocytosed upon cell activation. †Highly exocytosable ficolin-1-rich, gelatinase-poor granules found in neutrophils.

GO, Gene Ontology domains; MF, molecular function; BP, biological process; CC, cellular component.

UniProt IDs for proteins: refer to Supplemental Table 2.



No	GO	Over-represented pathways	p-value (adj)	-log$_{10}$ (p)
1	MF	Interleukin-1 receptor activity	0.00029	3.54
2	MF	Interleukin-1 binding	0.00049	3.31
3	MF	Carbohydrate binding	0.00503	2.30
4	MF	Interleukin-1, type I, activating receptor activity	0.03410	1.47
5	BP	Regulation of cellular extravasation	0.00412	2.38
6	BP	Regulation of interleukin-1-mediated signaling pathway	0.00526	2.28
7	BP	Cellular extravasation	0.00560	2.25
8	BP	Regulation of leukocyte migration	0.01989	1.70
9	BP	Neutrophil migration	0.03112	1.51
10	BP	Response to interleukin-1	0.04227	1.37
11	CC	Collagen-containing extracellular matrix	0.00084	3.08
12	CC	Extracellular matrix	0.00262	2.58
13	CC	External encapsulating structure	0.00264	2.58
14	CC	Cell periphery	0.00433	2.36
15	CC	Tertiary granule	0.00531	2.28
16	CC	Ficolin-1-rich granule	0.00761	2.12
17	CC	Ficolin-1-rich granule membrane	0.03677	1.43
18	CC	Membrane microdomain	0.04622	1.34
19	CC	Membrane raft	0.04622	1.34

Figure 6. Over-represented pathways in the plasma proteome of AS patients with diabetes.

Using the g:Profiler with Gene Ontology terms, functional enrichment analyses were performed with the proteomic analysis results. The size of each circle signifies intersection size.

adj, adjusted; AS, aortic stenosis; GO, Gene Ontology domains; MF, molecular function; BP, biological process; CC, cellular component.

3.4. Clinical outcomes according to the presence of diabetes

The participants in the imaging cohort was followed for a median 6.3 (IQR 5.2−7.2) years and nearly all patients (n=232, 91.7%) received AVR during follow−up. There were 53 events of unexpected admission for heart failure or death (20.9%) (Table 12).

Table 12. Number of clinical events in the entire population.

	Total (n=253)	Non−DM (n=187)	DM (n=66)	p−value (log−rank)
Admission for heart failure	18 (17.1)	8 (4.3)	10 (15.2)	<0.001
All−cause death	39 (15.4)	23 (12.3)	16 (24.2)	0.009
Composite of admission for heart failure and death	53 (20.9)	30 (16.0)	23 (34.8)	<0.001

DM, diabetes mellitus.

The incidence of the composite clinical events was significantly higher in diabetic AS patients compared to the non−diabetic AS subjects (Figure 7A); all−cause mortality was also higher in diabetic AS subjects (Figure 7B). Diabetes was a significant predictor of heart failure and all−cause death, independent of age, sex, atrial fibrillation, IHD, LV ejection fraction, and AVR (HR 1.88, 95% CI 1.06−3.31, p=0.030) (Table 13).

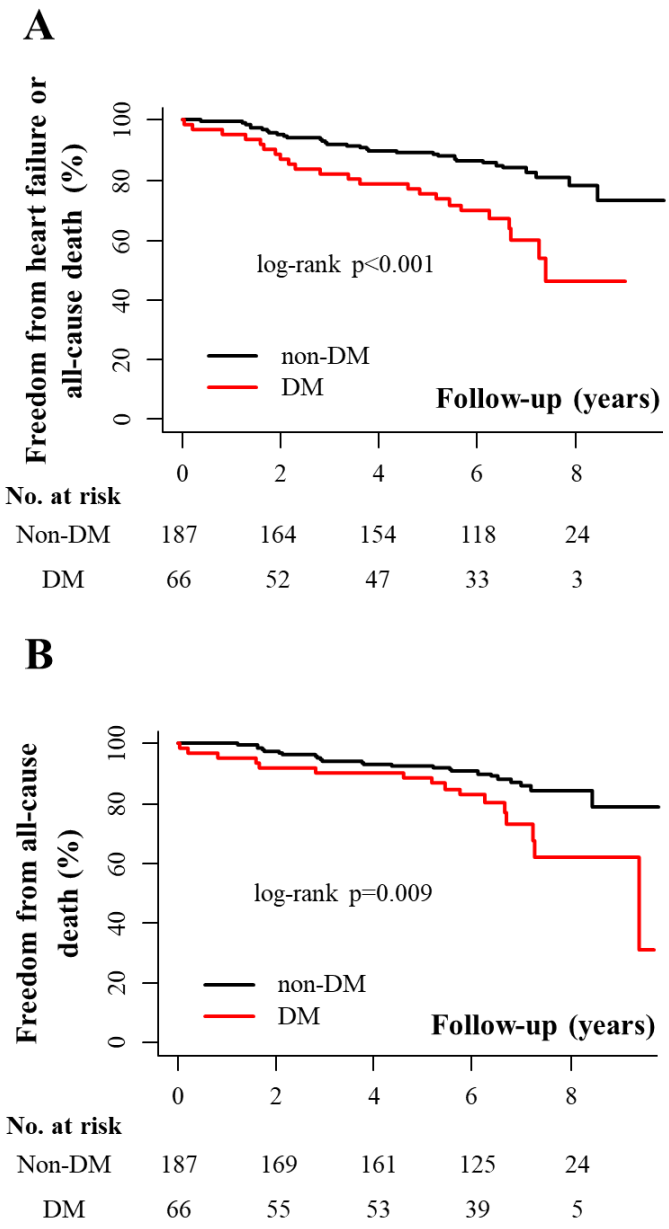


Figure 7. Comparison of event-free survival in AS patients according to diabetes.

Kaplan-Meier analysis with p-values by the log-rank test are presented for (A) the composite outcome of admission for heart failure and all-cause death, and (B) all-cause death only.

AS, aortic stenosis; DM, diabetes mellitus.

Table 13. Predictors of unexpected admission for heart failure or all-cause mortality.

	Univariable		Multivariable Model*	
	Crude HR	p-value	Adjusted HR	p-value
Age (years)	1.07 (1.03-1.11)	<0.001	1.06 (1.02-1.10)	0.003
Male	1.67 (0.96-2.89)	0.069	1.62 (0.92-2.85)	0.097
Diabetes	2.71 (1.57-4.68)	<0.001	1.88 (1.06-3.31)	0.030
Hypertension	1.57 (0.88-2.80)	0.125		
Atrial fibrillation	2.88 (1.56-5.30)	0.001	2.68 (1.42-5.07)	0.002
Stroke	1.39 (0.60-3.26)	0.445		
Ischemic heart disease	3.82 (2.14-6.83)	<0.001	2.48 (1.35-4.54)	0.003
Peak aortic velocity (m/s)	0.65 (0.47-0.89)	0.008		
AV replacement	0.33 (0.17-0.64)	0.001	0.22 (0.11-0.45)	<0.001
LV ejection fraction (%)	0.97 (0.95-0.99)	0.011	0.97 (0.95-0.99)	0.010

*Constructed with stepwise backward selection from variables presented in the first column.

AV, aortic valve; HR, hazard ratio; LV, left ventricular.

In the analysis of patients who underwent AVR (n=232), the AS patients with concomitant diabetes also had worse clinical outcomes (Figure 8, Table 14), again suggesting that diabetes has a pervasive systemic effect on the myocardial health even after relief of pressure overload by AVR.

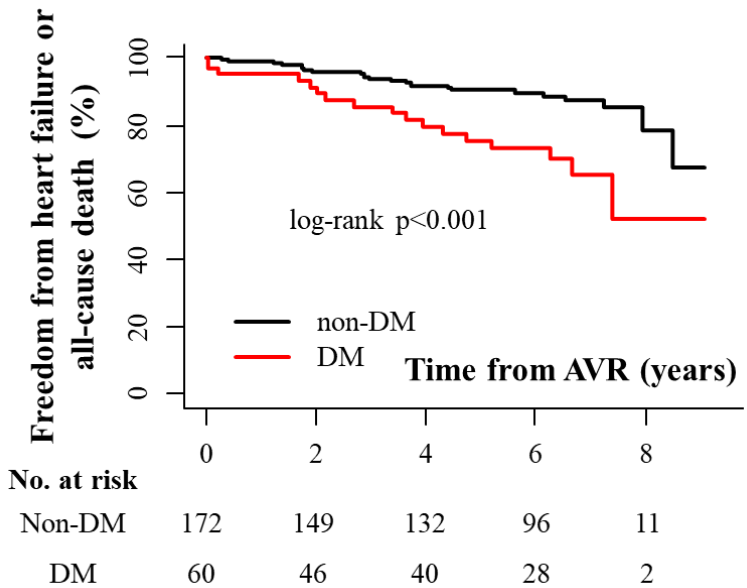


Figure 8. Comparison of event-free survival in AS patients according to diabetes, after AVR.

Kaplan-Meier analysis with p-values by the log-rank test are presented for the composite outcome of admission for heart failure and all-cause death.

AS, aortic stenosis; AVR, aortic valve replacement; DM, diabetes mellitus.

Table 14. Number of clinical events in the patients who underwent aortic valve replacement.

	Total (n=232)	Non-DM (n=172)	DM (n=60)	p-value (log-rank)*
Admission for heart failure	14 (6.0)	6 (3.5)	8 (13.3)	0.002
All-cause death	28 (12.1)	16 (9.3)	12 (20.0)	0.030
Composite of admission for heart failure and death	38 (16.4)	21 (12.2)	17 (28.3)	<0.001

*Index date as the date of aortic valve replacement.

DM, diabetes mellitus.

Chapter 4. Discussion

In the current study, we demonstrated that AS patients with diabetes compared to non-diabetic patients had increased diffuse interstitial and replacement fibrosis by CMR analysis of the myocardium. With an in-depth investigation of the plasma proteomics, factors related to proinflammatory response and extracellular matrix components were enriched in diabetic AS patients. These diabetic AS patients had a significantly higher incidence of heart failure and death than the non-diabetic patients, independent of other important clinical covariates. These results suggest that diabetes is associated with effects that potentiates the systemic proinflammatory and profibrotic milieu to the pressure-overloaded myocardium, which translate to worse clinical outcomes even after AVR.

4.1. Myocardial remodeling and poor prognosis in AS patients with diabetes

By using a combination of comprehensive noninvasive imaging modalities, we found that the degree of myocardial fibrosis by CMR and diastolic dysfunction by echocardiography is significantly more advanced in diabetic AS patients. There have been few studies on how diabetes impacts myocardial remodeling in patients with AS. An invasive histological study of myocardial specimens in 60 AS patients undergoing AVR suggested that patients with concomitant

AS and diabetes had increased myocardial fibrosis and higher cardiomyocyte stiffness.(17) Using comprehensive noninvasive imaging, we now show that the histologic evidence from the previous study(17) holds true in a larger AS population with a chance to examine the entire myocardium.

Studies utilizing CMR in the general population have also suggested that ECV and LGE are increased in diabetic compared to non-diabetic subjects, and that these measures are associated with future heart failure and mortality.(30-33) In AS patients, diabetes is independently associated with increase in both mid-term and long-term mortality in those undergoing AVR, as well as in those with conservatively managed asymptomatic AS.(19, 20, 34, 35) Herein, we provide the missing link between diabetes and outcome in AS patients by demonstrating the association between diabetes and myocardial fibrosis. Moreover, the need for more intensive diabetes treatment as a marker of diabetes severity was associated with greater degree of myocardial fibrosis, as shown by the highest measures of fibrosis as well as LV diastolic dysfunction in the insulin-treated diabetic patients. In the pressure-overloaded heart, myocardial fibrosis is an important driver of the progression from compensated hypertrophy to heart failure with diastolic and systolic dysfunction, findings demonstrated in both histological and imaging studies.(36-38) In the current study, AS patients with diabetes had greater replacement and diffuse interstitial fibrosis compared to non-diabetic counterparts (presence of LGE 56% vs 40%, p=0.036; LGE% 1.2 [0.4-2.9] vs. 0.6 [0.2-1.5] in patients with

LGE, p=0.009; ECV% 27.9 [25.7−30.1] vs. 26.7 [24.9−28.5], p=0.025). In AS patients, presence of myocardial replacement fibrosis has been independently associated with 2.4−fold higher mortality, and each 1% increase in LGE and ECV with 11% and 10% higher mortality, respectively.(6, 7) Furthermore, LGE and ECV seem to have a non−linear association with outcome, and even a small increase from the normal range can lead to significantly worse outcomes.(8)

Previous studies have shown that the prognosis of diabetic AS patients even after AVR are significantly worse especially in those treated with insulin,(20, 34, 35) which supports that factors other than the stenotic valve in AS is responsible for the worse prognosis in diabetic AS patients. Diabetes is also associated with poor LV mass regression after AVR,(18) suggesting that diabetes continues to affect myocardial remodeling after relief of pressure overload. Our findings provide a missing link as to diabetes aggravates the prognosis of AS patients, most probably by influencing the myocardial health and its remodeling. This also lead us to question whether and how diabetes changes the systemic milieu and ultimately, the myocardium.

4.2. Systemic proinflammatory and profibrotic response as the main pathophysiological process in AS patients with diabetes

Circulating protein biomarkers provide important information on

pathophysiological mechanisms of diseases, and high-throughput proteomic methods can measure a multitude of proteins simultaneously.(39) In previous plasma proteome studies of AS patients, higher GDF-15 was associated with poor LV reverse-remodeling and increased mortality after AVR.(40, 41) In plasma proteome analysis of patients with heart failure, diabetic patients had higher circulating GDF-15 and galectin-4 levels,(42) and over-representation of pathways related to inflammation, cardiac remodeling, and fibrosis.(42, 43) We found that E-selectin, interleukin-1 receptor type 1, interleukin-1 receptor type 2, galectin-3, galectin-4, intercellular adhesion molecule 2, integrin beta-2, GDF-15, and cathepsin D levels were significantly upregulated in diabetic AS patients, proteins which have been implicated in inflammation, cardiac fibrosis and remodeling, atherosclerosis, and heart failure.(44-51) Furthermore, over-representation analyses of the plasma proteome demonstrated that pathways related to neutrophil activation, interleukin-1 and amplification of inflammation, leukocyte migration, and extracellular matrix were enriched in diabetic AS patients. Our study supports that systemic upregulation of signals related to inflammation and extracellular matrix expansion are important pathophysiological processes mediated by diabetes in AS patients systemically, which in turn, may aggravate the degree of myocardial fibrosis and lead to worse outcomes.

4.3. Clinical implications and future direction

Our study suggests that clinicians should be aware of the significantly higher clinical events in AS patients with diabetes. According to our analysis, diabetes not only damages the stenotic valve,(12-15) but also, the health of the myocardium, with its effect prevailing even after AVR. Although we do not have data on how the myocardium changes after AVR, our findings suggest that the changes in the myocardium by AS are already more advanced in diabetes patients and it can easily be assumed that the systemic proinflammatory and profibrotic milieu will continue with diabetes even after AVR. This also suggests that AVR itself is not the ultimate treatment for AS when the patient has diabetes.

Considering the systemic proinflammatory and profibrotic environment by diabetes, our findings call for further studies on whether anti-inflammatory approaches may be beneficial in AS patients with concomitant diabetes. Exciting options testing this idea, have been developed recently with promising outcomes, such as the monoclonal antibody targeting interleukin-1β.(52) Furthermore, currently used anti-diabetic medication may alleviate inflammation by differing degrees,(53) which may also affect diabetes-related myocardial remodeling and fibrosis, especially in the pressure-overloaded myocardium of AS patients. This should be tested in the near future with animal studies as well as in clinical trials.

4.4. Study limitations

Our study is not without limitations. First, because of the shortage of resources, two separate imaging and biomarker cohorts were used for analysis, and analysis of the direct association of plasma proteins with noninvasive measures of fibrosis on CMR could not be performed. Second, information on the duration of diabetes and control of diabetes assessed by HbA1c values was not available, which can influence the impact of diabetes on the myocardium. Thus, we used diabetes medication status as a surrogate marker of the severity and chronicity of diabetes, but its limitations must be acknowledged. Third, myocardial dysfunction in the systemic diabetic milieu involves myocyte dysfunction as well as progression of extracellular fibrosis. We used CMR techniques for the in-depth evaluation of the myocardium, but these methods mainly focus on extracellular myocardial fibrosis and cannot assess cellular dysfunction such as increased myocyte stiffness and impaired myocardial energetics. Lastly, we used a select biomarker panel of 92 proteins, and future studies utilizing a more comprehensive set of proteins may provide more pathophysiological information as well as therapeutic targets.

4.5. Conclusions

Plasma proteome analyses indicate that diabetes is associated with increased systemic proinflammatory and profibrotic responses in patients with AS. These biological changes underlie the increase of

myocardial fibrosis, more advanced LV diastolic dysfunction, and ultimately, worse clinical outcomes observed in patients with concomitant AS and diabetes.

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국문 초록

대동맥판막협착증 환자에서 당뇨에 의한 혈장단백체의 변화 및 심근의 재형성과 예후와의 관련성 연구

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연구 배경: 대동맥판막협착증에 당뇨병이 동반된 경우, 당뇨병이 심근의 재형성 및 섬유화에 미치는 영향 및 그 기전에 대하여 정확히 밝혀지지 않았다. 본 연구에서는 당뇨병이 대동맥판막협착증 환자들의 예후에 미치는 영향 및 그 기전에 대하여 비침습적인 영상검사 및 혈장 단백체학(proteomics)을 포괄적으로 이용하여 연구하고자 하였다.

연구 방법: 본 연구는 영상검사 코호트와 바이오마커 코호트를 활용하여 진행하였다. 영상검사 코호트에는 심초음파와 심장자기공명영상을 같이 시행한 중증 대동맥판막협착증 환자 253명(그 중 당뇨 66명)이 포함되었으며, 심근의 대치성 섬유화(replacement fibrosis)와 미만성 간질성 섬유화(diffuse interstitial fibrosis)의 정도를 심장자기공명영상 분석을 통하여 각각 가돌리늄 지연 조영증강(late gadolinium enhancement; LGE) 및 extracellular volume fraction(ECV)으로 정량적으로 측정하였다. 바이오마커 코호트에는 혈액샘플을 채취한 100명의 중증 대동맥판막협착증 환자들(그 중 당뇨 27명)이 포함되었으며, 다중 근접 연장 측정법 (multiplex proximity extension assay)를 이용하여 혈장의 단백체 분석을 진행하였다.

연구 결과: 영상검사 코호트에서 당뇨병이 동반된 대동맥판막협착증 환자들은 비당뇨병 환자들에 비하여 나이가 더 많고 (70.4±6.8 vs. 66.7±10.1세) 허혈성심장질환의 빈도가 더 높았으며 (28.8% vs. 9.1%), 심초음파 상 더 진행된 좌심실 이완기능 장애를 보였다. 심장자기공명영상에서 당뇨병 환자들은 심근의 대치성 및 간질성 섬유화 모두 증가된 소견이 확인되었다 (LGE% 0.3 [0.0–1.6] vs. 0.0 [0.0–

0.5], p=0.009; ECV% 27.9 [25.7−30.1] vs. 26.7 [24.9−28.5], p=0.025). 바이오마커 코호트의 혈장 단백체 분석을 통하여 당뇨병이 동반된 대동맥판막협착증 환자들의 혈중에서 9개의 단백질(E−selectin, interleukin−1 receptor type 1, interleukin−1 receptor type 2, galectin−4, intercellular adhesion molecule 2, integrin beta−2, galectin−3, growth differentiation factor 15, and cathepsin D)이 유의하게 증가되어 있음을 확인하였다. Gene ontology terms를 이용한 혈장 단백체의 과발현 경로 분석 (pathway over−representation analysis) 시, 염증 반응 및 세포외기질과 관련된 경로들의 발현이 유의하게 증가한 것으로 나타났다. 이러한 결과는 당뇨병이 전신적인 효과를 통하여 압력 과부하 상태인 심근에서 염증 및 섬유화를 증가시키는 역할을 한다는 것을 시사한다. 영상검사 코호트를 6.3년간 (중앙값 6.3년, 사분위수범위 5.2−7.2년) 추적 관찰하였을 때, 232명(91.7%)이 대동맥판막치환술을 받았으며 심부전으로 인한 입원 또는 사망이 53명 있었다. 당뇨병은 다른 임상적 공변량들이나 대동맥판막치환술의 여부와 무관하게 심부전 및 사망의 발생의 유의한 예측인자로 확인되었다.

결론: 혈장 단백체 분석에 따르면 당뇨병은 대동맥판막협착증 환자들에서 전신적인 염증 및 섬유화 반응을 강화시킨다. 이러한 전신적 생물학적인 변화는 당뇨병이 동반된 대동맥판막협착증 환자들에서 나타나는 심근 섬유화의 증가, 이완기능장애의 진행 및 불량한 예후의 기저에 있다.

주요어: 대동맥판막협착증, 당뇨병, 자기공명영상, 심초음파, 단백체
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