Circulation

ORIGINAL RESEARCH ARTICLE

Proteomic Biomarkers for Incident Aortic Stenosis Requiring Valvular Replacement

BACKGROUND: Aortic valve stenosis (AS) is the most common indication for cardiac valve surgery; untreated AS is linked to high mortality. The etiological background of AS is unknown. Previous human studies were typically based on case-control studies. Biomarkers identified in prospective studies could lead to novel mechanistic insights.

METHODS: Within a large population survey with blood samples obtained at baseline, 334 patients were identified who later underwent surgery for AS (median age [interquartile range], 59.9 [10.4] years at survey and 68.3 [12.7] at surgery; 48% female). For each case, 2 matched referents were allocated. Plasma was analyzed with the multiplex proximity extension assay for screening of 92 cardiovascular candidate proteins. Conditional logistic regression models were used to assess associations between each protein and AS, with correction for multiple testing. A separate set of 106 additional cases with 212 matched referents was used in a validation study.

RESULTS: Six proteins (growth differentiation factor 15, galectin-4, von Willebrand factor, interleukin 17 receptor A, transferrin receptor protein 1, and proprotein convertase subtilisin/kexin type 9) were associated with case status in the discovery cohort; odds ratios ranged from 1.25 to 1.37 per SD increase in the protein signal. Adjusting the multivariable models for classical cardiovascular risk factors at baseline yielded similar results. Subanalyses of case-referent triplets (n=133) who showed no visible coronary artery disease at the time of surgery in the index person supported associations between AS and growth differentiation factor 15 (odds ratio, 1.40; 95% confidence interval, 1.10–1.78) and galectin-4 (odds ratio, 1.27; 95% confidence interval, 1.02–1.59), but these associations were attenuated after excluding individuals who donated blood samples within 5 years before surgery. In triplets (n=201), which included index individuals with concurrent coronary artery disease at the time of surgery, all 6 proteins were robustly associated with case status in all sensitivity analyses. In the validation study, the association of all but 1 (interleukin 17 receptor A) of these proteins were replicated in patients with AS with concurrent coronary artery disease but not in patients with AS without coronary artery disease.

CONCLUSIONS: We provide evidence that 5 proteins were altered years before AS surgery and that the associations seem to be driven by concurrent atherosclerotic disease.

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Clinical Perspective

What Is New?

- This study provides evidence that the biochemical risk profile differs in aortic stenosis (AS) subtypes.
- Five circulating proteins altered years before surgery were associated with AS only in patients with concomitant coronary artery disease.

What Are the Clinical Implications?

- Most pharmacological interventions for AS have been unsuccessful. The different phenotypes of AS must be taken into account when designing intervention trials for AS.
- The identified 5 proteins could reveal new insights in the pathophysiology behind the development of the most common subtype of AS.

Aortic valvular replacement (AVR) because of aortic valve stenosis (AS) is the second most common thoracic surgery procedure in Western countries.[1] The prevalence of AS is 2% to 4% in the general population and higher in older age groups.[1] The disease develops slowly, with thickening and calcification of the valve and narrowing of the outflow of the left ventricle. Symptoms occur after several years of disease development. According to guidelines, patients with asymptomatic AS should undergo regular clinical exams, and AVR is recommended in patients with severe AS, with either reduced left ventricular function or symptoms related to stenosis.[1] The fatality is ≈50% within 2 years of symptom onset when no surgery is performed.[1]

The etiology of calcification is unknown, but the aging process, inflammatory mechanisms, and atherosclerosis have been suggested as possible factors because atherosclerotic lesions and valvular calcification have similar histologies and associations with traditional cardiovascular risk factors.[1] Previously, the pathological process was regarded as a passive, degenerative process, but it is currently thought to be an active biological process with histological evidence of inflammation and extracellular matrix remodeling, which lead to ectopic bone formation.[1]

In cross-sectional studies, traditional cardiovascular risk factors, such as hypertension and total cholesterol, have been associated with AS and a few biomarkers, including lipoprotein (a), brain natriuretic peptide, and galectin-3.[1–4] However, many of those studies did not stratify the study population to examine the influence of concomitant coronary artery disease (CAD).

In this study, we identified all cases of incident AVR within a large ongoing population-based study in northern Sweden. With a prospective case-referent design, including a validation cohort, we aimed to identify novel protein biomarkers for incident AS. We also hypothesized that these associations might differ when the concomitant presence of CAD was considered.

METHODS

Study Population

A total of 6691 patients underwent surgery for valvular heart disease or disease of the ascending aorta in the Department of Cardiothoracic Surgery, Umeå University Hospital, Sweden, between March 1988 and December 2014. Before their first surgery, 708 of these patients had participated in 1 of 3 population-based health studies within the Northern Sweden Health and Disease Study, and they had donated blood for future research. Among these patients, 334 had later undergone surgery for AS (between October 1992 and April 2012). We retrieved plasma samples for each of these 334 patients, including 235 samples (70%) from the VIP (Västerbotten Intervention Program), 37 samples (11%) from the MONICA survey (Northern Sweden Monitoring of Trends and Determinants in Cardiovascular Disease), and 62 samples (19%) from the MSP (Mammary Screening Program).

VIP is an ongoing community intervention program with the aim of preventing cardiovascular disease and diabetes mellitus. In this program, all county residents at 30 (until 1995), 40, 50, and 60 years of age were asked to participate in a health survey and receive health counseling at their primary healthcare center.[5] MONICA enrollment involved asking randomly selected individuals to participate in a health survey. Participants were 25 to 74 years of age, and they resided in the counties of Västerbotten and Norrbotten.[6] The MSP cohort comprised women who attended routine mammography screenings. Taken together, these 3 surveys included 140 414 participants, up to December 2014, which reflected participation rates of 65% to 75%.

For each case, we randomly selected 2 referents (controls) who were matched for sex, age (±2 years), type of survey (MONICA, VIP, or MSP), date of health survey (±4 months), and geographic area. However, in the discovery cohort, 6 matched sets contained only 1 referent each because of a lack of individuals fulfilling the matching criteria. We did not exclude referents or cases (patients) with a history of myocardial infarction or cancer before the survey. In the cohort, 2.7% of cases and 3.3% of referents had been diagnosed with cancer within 5 years before surgery (or the corresponding date for referents). These proportions were 0.9% and 1.1%, respectively, after exclusion of those triplets where the index person had visible CAD at surgery. Similarly, 2.4% of cases and 1.4% of referents reported a prior myocardial infarction; these proportions were 0.3% and 0.5%, respectively, after excluding those triplets where the index person had visible coronary atherosclerosis at surgery.

For validation of the findings, 106 new cases with AVR surgery for AS were identified within the cohorts: 70 from VIP (66%), 16 from MONICA (15%), and 16 from MSP (19%). The majority of procedures were performed between April 2012 and December 2014.

The study protocol was approved by the Regional Ethics Review Board in Umeå (dnr 2014-348-32M and 07-174M), and it complied with the Declaration of Helsinki. All participants

provided written informed consent for future use of the data and samples.

The dataset generated during the current study is not publicly available because it contains identifiable patient data but is available from the corresponding author on reasonable request.

Perioperative Characteristics

From hospital files, we acquired data on preoperative assessments, including the medical history, current medication, anthropometry, blood pressure, ECG, coronary angiogram, and echocardiography, when available. We also recorded perioperative details, such as the nature of valvular disease (eg, malformations, calcification, and endocarditis), type of valvular intervention (eg, mechanical or biological prosthesis or valvuloplasty), and the number of coronary grafts. According to established practice, all except 1 case in the discovery cohort and 1 in the validation cohort underwent a coronary angiogram, and any atheromatosis was taken to indicate CAD (found in 201 patients [60.4%] in the discovery cohort and in 65 patients [61.9%] in the validation cohort).

All 334 patients in the discovery cohort received an AVR because of AS. In 84% of patients, the primary indication was AS; the remaining 16% received aortic valvular surgery combined with another primary intervention, such as coronary artery bypass surgery (10%) or surgery for ascending aortic disease (5%). Similarly, all 106 patients in the validation cohort received an AVR, and the primary indications were AS in 88%, coronary artery bypass surgery in 5%, or surgery for the ascending aorta in 5%.

The patient characteristics at surgery are summarized in Table I in the online-only Data Supplement. Patients with concomitant CAD were more often men and were older at both survey and surgery. They more often had a history of a previous myocardial infarction and coronary bypass surgery. They had higher systolic blood pressure, and their aortic valves were less stenotic with lower gradients. The same pattern was seen in the validation cohort.

Baseline Clinical Examinations and Biochemical Analysis

We have recently thoroughly described clinical examinations and biochemical analysis performed at baseline.[7,8] During the initial health survey, participants in VIP and MONICA were asked to complete a health questionnaire regarding their living conditions and cardiovascular risk factors. These participants also underwent anthropometry and blood pressure measurements. Participant weight was measured in light, indoor clothing without shoes and was recorded to the nearest 0.2 kg. Height was measured without shoes to the nearest centimeter. Body mass index was calculated. Subjects were categorized by whether they were smokers, were ex-smokers, or had never smoked tobacco (never smokers).

An oral glucose tolerance test was performed, and glucose tolerance categories were defined according to World Health Organization guidelines.[9] Glucose intolerance was defined as impaired fasting glucose and impaired glucose tolerance or diabetes mellitus.

In the MONICA and MSP surveys, blood pressure was recorded in the sitting position after 5 minutes of rest. Initially, a mercury sphygmomanometer was used, but starting in 2004, semiautomatic devices were used (Omron M7, Omron Corp). In the VIP survey, blood pressure was measured after 5 minutes of rest in the recumbent position until September 1, 2009; thereafter, it was measured in the sitting position with the devices described earlier. Measurements obtained with participants in the recumbent position were adjusted with a sex- and age-specific formula.[10] Hypertension was defined as a systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, and use of antihypertensive medication.

Total serum cholesterol was measured at the time of the health survey with a bench-top analyzer (Reflotron, Boehringer Mannheim GmbH Diagnostica) in the VIP until September 2009. In the VIP (after September 2009) and MONICA surveys, serum cholesterol was measured at a central laboratory with an enzymatic method (Boehringer Mannheim GmbH Diagnostica). Cholesterol values obtained with the bench-top method were adjusted to facilitate comparisons with the results measured at the central laboratory. Plasma EDTA samples were obtained after fasting for ≥4 hours (extended to 8 hours after 1992). The samples were aliquoted and deep frozen within 1 hour and then transferred to the Northern Sweden Biobank, where they were stored at –80°C until analysis.

In 2017, apolipoprotein (Apo) A1 and ApoB were analyzed in the discovery cohort on a Cobas 8000 modular analyzer, c502 module. The reagents used were Tina-quant ApoA1 and B (Cat. Nos. 03032566122 and 03032574122, respectively, version 2, Roche Diagnostics). ApoA1 and ApoB were standardized to reference standards, The International Federation of Clinical Chemistry and Laboratory Medicine SP1-01 and SP3-07, respectively. The ApoB/A1 ratio was calculated. The total coefficients of variation were ApoA1: 3.42% and 2.18% at levels of 0.86 and 1.45 mg/L, respectively; and ApoB: 1.93% and 2.19% at levels of 1.0 and 1.8 mg/L, respectively.

Proteomic Profiling and Quality Control

Plasma samples were analyzed at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala University, with the proximity extension assay (PEA) technique using the Olink Multiplex CVD III 96×96 panel, a high-specificity assay that simultaneously measures concentrations of 92 cardiovascular candidate proteins.[11,12] In brief, the assay uses a standard 96-well microplate format, including 90 samples and 6 external quality control standards. Each sample is mixed with 92 pairs of oligonucleotide-labeled antibodies and 4 internal technical controls. When both high-specificity antibodies bind the target protein in the sample, the attached oligonucleotides form a unique DNA reporter sequence that is subsequently amplified and quantified with a standard polymerase chain reaction. Samples were analyzed in individual wells on 12 plates, and each case and its 2 referents were always analyzed in the same plate. All samples were randomly distributed in the plates. Polymerase chain reaction values above the fluorescence detection threshold were log2-transformed and corrected for technical variations based on negative and interplate controls. The lower limits of detection were determined with the negative control samples (buffer without antigen). In the quality control procedure, 2 proteins (SPON1 and

N-terminal pro B-type natriuretic peptide) had >5% missing values, and thus they were removed. For all 996 individuals in the dataset, the measurements were above the limits of detection in >97% of the remaining 90 proteins, and thus they were analyzed further. A principal components analysis indicated that the protein measurements were associated with plate and storage time.

In the validation cohort, only results from the 6 proteins identified as associated with AS in the discovery cohort were considered. In the quality control procedure, all 6 proteins had <2% missing values.

In both the discovery and validation cohorts, we used standardized residuals from linear regression models for each protein, adjusted for plate and storage time, as the measurement of protein variation.

Statistical Methods

We used STATA 14.1 for statistical analyses and R v 3.1.3 for visualization.

Initial Analysis

We used a series of nonadjusted conditional logistic regressions in the full discovery cohort to estimate the association between each protein and the case status with the *clogit* command in STATA. We used a 5% false discovery rate according to the method described by Benjamini and Hochberg[13] to determine significant associations.

Multivariable Adjustment and Stratified Analysis

For proteins associated with case status at a 5% false discovery rate, we performed a set of adjusted conditional logistic regression models. First, we used the complete-case approach, which excluded individuals with missing data, and the model was adjusted for hypertension status, smoking habits, glucose intolerance, and the plasma ApoB/A1 ratio. We then used multiple imputation by chained equation, with 20 iterations, to impute the missing values in hypertension status, smoking habits, glucose intolerance, ApoB/A1 ratio, and body mass index based on the complete-case information for these variables and education status, study center, age, and sex of the individual. The imputation thus included some of the matching variables but excluded the identifier of matched pairs, consistent with the method described by Seaman and Keogh.[14] The numbers of imputed values are shown in Table II in the online-only Data Supplement. Conditional logistic regression models were adjusted with coefficients and standard errors for the variability between imputations, according to the combination rules by Rubin.[15] These models were run for each of the 6 proteins, with adjustments for hypertension status, smoking habits, glucose intolerance, and ApoB. Furthermore, we stratified the analysis based on the presence/absence of atherosclerotic lesions and based on whether AS was the primary indication for surgery.

Sensitivity Analysis

In the sensitivity analysis, we restricted the combined sample by excluding the MSP dataset, which contained the majority of cases with missing data on covariates. We further restricted the analysis to individuals who had provided samples >5 years before surgery to avoid confounding with disease severity. We also included models adjusted for body mass index.

Validation Cohort

For those proteins identified as associated with AS status in the discovery cohort, the analyses were repeated in the validation cohort using the identical setting with the exception for the adjustment for the plasma ApoB/A1 ratio, which was replaced with total cholesterol adjustment.

RESULTS

Descriptives

The final discovery cohort included 334 cases and 662 referents (median age [interquartile range] at baseline, 59.8 [10.3] years, at surgery 68.3 [12.7] years; 48% women). The interval between survey and surgery was 10.8 (9.3) years. The validation cohort included 106 cases and 212 referents (median age at baseline, 59.8 [10.5] years, at surgery 72.8 [13.2] years; 45% women). The interval between survey and surgery was 16.6 (7.4) years. The baseline characteristics at survey are summarized in the Table.

Initial Analysis

After correcting for multiple testing in the conditional logistic regression analyses, 6 proteins were found to be associated with incident surgery for AS: growth differentiation factor 15 (GDF-15), galectin-4, von Willebrand factor (vWF), interleukin 17 receptor A, transferrin receptor protein 1 (TR), and proprotein convertase subtilisin/kexin type 9 (PCSK9) (Table III in the online-only Data Supplement). All these proteins were positively correlated with case status; the odds ratios (ORs) ranged from 1.25 to 1.37 per SD increase in protein signal.

In the validation cohort, GDF-15, galectin-4, vWF, and PCSK9 remained positively associated with case status; the ORs ranged from 1.37 to 1.68 per SD increase in protein signal (Table IV in the online-only Data Supplement).

Nonstratified Analysis: Multivariable Adjustment

All 6 proteins were also associated with surgery for AS in models adjusted for hypertension status, smoking habits, glucose intolerance, and the plasma ApoB/ApoA1 ratio in the full sample with imputed variables. ORs were largely unchanged from the initial analysis, which indicated that no major confounding was present. When we used the complete-case approach, where only individuals with complete information on all covariates were included, the sample size was reduced to about half. Nevertheless, in adjusted models, we observed significant associations ($P<0.05$) between surgery for AS and 5 of the 6 proteins (GDF-15, interleukin 17 receptor A, PCSK9, vWF, and TR).

After imputation and adjustments (hypertension status, smoking habits, glucose intolerance, cholesterol

Table. Basal Characteristics for the Discovery and Validation Cohorts

Variable	Discovery	Referents	Cases	*P* Value	Validation	Referents	Cases	*P* Value
N		662	334			212	106	
Age at survey, y	662/334	56.7 (56.1–57.4)	56.7 (55.8–57.6)	Matched	212/106	56.0 (54.7–57.3)	56.1 (54.3–57.9)	Matched
Age at surgery, y	—/334	—	67.2 (66.3–68.2)	—	—/106	—	71.5 (69.6–73.4)	—
Female sex, %	662/334	48.0 (44.2–51.9)	47.9 (42.5–53.3)	Matched	212/106	45.3 (38.5–52.0)	45.3 (35.7–54.9)	Matched
BMI, kg/m^2	646/320	26.1 (25.8–26.4)	26.9 (26.4–27.4)	0.01	200/102	25.8 (25.3–26.3)	27.8 (26.9–28.6)	<0.001
Cholesterol, mmol/L	526/263	6.2 (6.1–6.3)	6.4 (6.2–6.5)	0.06	164/80	6.0 (5.8–6.1)	6.2 (6.0–6.5)	0.09
ApoB/A1, ratio	640/308	0.81 (0.79–0.82)	0.85 (0.82–0.87)	0.02				
Hypertension, %	536/267	49.1 (44.8–53.3)	61.0 (55.2–66.9)	0.001	168/83	46.4 (38.8–54.1)	49.4 (38.4–60.4)	0.66
Glucose intolerance, %	481/240	19.8 (16.2–23.3)	26.7 (21.0–32.3)	0.04	152/69	25.7 (18.6–32.7)	34.8 (23.3–46.3)	0.18
Systolic BP, mm Hg	536/268	136 (134–137)	138 (136–141)	0.04	168/83	133 (130–135)	136 (131–139)	0.28
Diastolic BP, mm Hg	536/267	84 (84–85)	86 (85–87)	0.04	168/83	84 (83–85)	83 (81–85)	0.38
Education (n)	512/255			0.45	146/73			0.67
Lower compulsory school		361	189			98	45	
Secondary school		65	32			27	7	
University		86	34			21	11	
Smoking (n)	522/256			0.29	162/85			0.03
Nonsmoker		241	103			81	28	
Ex-smoker		177	95			56	37	
Smoker		104	58			25	20	

Data shown are findings from the health survey, stratified for cohort and case status. Available numbers and means with 95% confidence intervals are presented. *P* values were based on the Student's *t* test or the χ^2 test when appropriate. Apo indicates apolipoprotein; BMI, body mass index; and BP, blood pressure.

levels), GDF-15, galectin-4, vWF, and PCSK9 associated with future AVR in the validation cohort. Using the complete-case approach, galectin-4 and PCSK9 remained associated with AVR. Similar to the discovery cohort, the sample size was reduced to about half.

Stratified Analysis

No CAD

When the analysis was stratified based on the presence/absence of CAD at the time of surgery, we found evidence for associations between AS without CAD and GDF-15 in the discovery cohort (OR, 1.40; 95% confidence interval, 1.10–1.78) and galectin-4 (OR, 1.27; 95% confidence interval, 1.02–1.59). Adjusted models yielded similar estimates but with slightly larger confidence intervals (GDF-15: OR, 1.33; 95% confidence interval, 1.03–1.72; galectin-4: OR, 1.25; 95% confidence interval, 1.00–1.57).

GDF-15 and galectin-4 did not associate with future AVR in those without CAD in the validation cohort. The results are shown in Figure 1A for the discovery and the validation cohorts.

Concomitant CAD

Six proteins were associated with future AVR in the presence of concomitant CAD in nonadjusted and adjusted models. Five of them, GDF-15, galectin-4, vWF, TR, and PCSK9, were also found associated with incident AVR with CAD in the validation cohort. Further adjustment for body mass index did not change these associations. The results are shown in Figure 1B for the discovery and validation cohorts.

Sensitivity Analysis

Restricting the analysis to the VIP and MONICA cohorts (272 cases and 538 referents remained) yielded results similar to those obtained in the main analysis (data not shown). Furthermore, we conducted another analysis that included only individuals who had provided blood samples >5 years before surgery (260 cases and 514 referents remained). In this analysis, the previously identified associations between nonatherosclerotic AS and GDF-15 and galectin-4 were less prominent (Figure 2A), but associations between atherosclerotic AS were evident for all 6 proteins (Figure 2B). Further stratification for time between sampling and surgery did not indicate any trends in risk estimates with time for any of the 6 proteins (data not shown).

In the validation cohort, restricting the analysis to the VIP and MONICA cohorts (86 cases and 172 referents remained) yielded similar results to the main analysis (data not shown). Furthermore, GDF-15, galectin-4, vWF, TR, and PCSK9 remained associated with AS with CAD after

A No CAD

	Unadjusted	Adjusted	Validation unadj.	Validation adj.
GDF15	1.40 (1.10, 1.78)	1.33 (1.03, 1.72)	0.72 (0.40, 1.31)	0.69 (0.36, 1.33)
Gal4	1.27 (1.02, 1.59)	1.25 (1.00, 1.57)	1.10 (0.72, 1.68)	1.23 (0.76, 2.01)
IL17RA	1.21 (0.98, 1.50)	1.19 (0.96, 1.48)	1.09 (0.70, 1.70)	1.02 (0.63, 1.67)
PCSK9	1.10 (0.90, 1.35)	1.11 (0.90, 1.38)	1.48 (0.95, 2.31)	1.46 (0.88, 2.41)
TR	1.10 (0.89, 1.34)	1.11 (0.91, 1.37)	0.95 (0.64, 1.41)	0.97 (0.63, 1.50)
vWF	1.13 (0.87, 1.47)	1.07 (0.82, 1.40)	1.15 (0.74, 1.78)	1.07 (0.66, 1.73)

B CAD

	Unadjusted	Adjusted	Validation unadj.	Validation adj.
GDF15	1.39 (1.15, 1.69)	1.42 (1.15, 1.75)	1.83 (1.23, 2.73)	1.60 (1.03, 2.49)
Gal4	1.30 (1.08, 1.56)	1.27 (1.05, 1.55)	1.60 (1.16, 2.21)	1.65 (1.15, 2.37)
IL17RA	1.32 (1.09, 1.60)	1.38 (1.13, 1.69)	1.05 (0.77, 1.43)	1.05 (0.76, 1.46)
PCSK9	1.44 (1.18, 1.76)	1.46 (1.18, 1.80)	1.82 (1.28, 2.61)	1.81 (1.23, 2.67)
TR	1.41 (1.15, 1.72)	1.41 (1.14, 1.74)	1.40 (0.99, 2.00)	1.60 (1.07, 2.39)
vWF	1.37 (1.13, 1.67)	1.44 (1.17, 1.78)	1.63 (1.14, 2.31)	1.60 (1.08, 2.36)

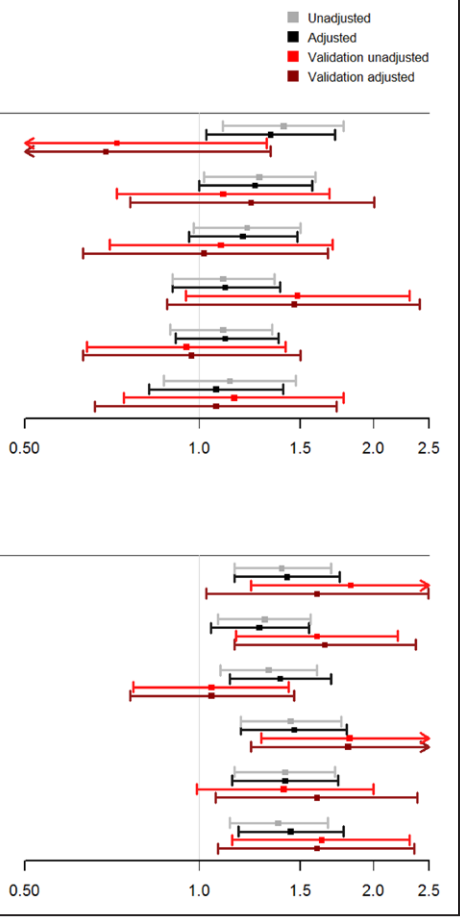

Figure 1. Associations between future surgery for aortic stenosis (AS) and 6 plasma proteins.
A, Associations with AS in the absence of concomitant coronary artery disease (CAD). **B**, Associations with AS in the presence of concomitant CAD. Unadjusted and adjusted estimates are shown in gray and black for the discovery cohort and in red and brown for the validation cohort. GDF-15 indicates growth differentiation factor 15; Gal4, galectin-4; IL17RA, interleukin 17 receptor A; PCSK9, proprotein convertase subtilisin/kexin type 9; TR, transferrin receptor protein 1; and vWF, von Willebrand factor.

exclusion of those with surgery within 5 years from survey (100 cases and 200 referents remained) (Figure 2B).

To assess the linear relationship between proteins and log odds of the AS and the identified proteins, cubic spline models were fitted for associated proteins.

No evidence of deviance from a linear relationship of the associations based on visual inspection of plots of predicted risk from cubic spline models across the protein levels was detected (data not shown).

DISCUSSION

The results of the present investigation suggested that alterations in 4 proteins—GDF-15, galectin-4, PCSK9, and vWF—were associated with later development of AS that required valvular replacement. These associations were significant after adjusting for established risk factors. Two of these proteins, GDF-15 and galectin-4, were also found to be produced at elevated levels in patients without visible CAD in the discovery cohort but not in the validation cohort. Additionally, levels of TR were associated with future AVR with concurrent CAD in both discovery and validation cohorts.

In previous studies, high circulating levels of GDF-15, PCSK9, and vWF have been associated with both prevalent and incident CAD.[16–18] There is no known evidence linking TR and galectin-4 with the development of CAD. However, associations have been suggested for other members of the galectin family of proteins.[19,20] There is good reason to believe that, to some extent, the changes in expression of at least 3 of the 5 proteins, PCSK9, TR, and vWF, could be explained by the prevalence of CAD, and thus they might not be specific predictors of aortic valve pathology.

GDF-15 is a stress-responsive cytokine that is highly expressed in cardiomyocytes, endothelial cells, and vascular smooth muscle cells in normal and pathological conditions. GDF-15 is strongly upregulated in response to biomechanical stress on cardiomyocytes, and it is a predictor of heart failure development.[21,22] Elevated GDF-15 levels were previously found in patients with AS, and it was proposed to be a prognostic marker for survival after surgery.[23]

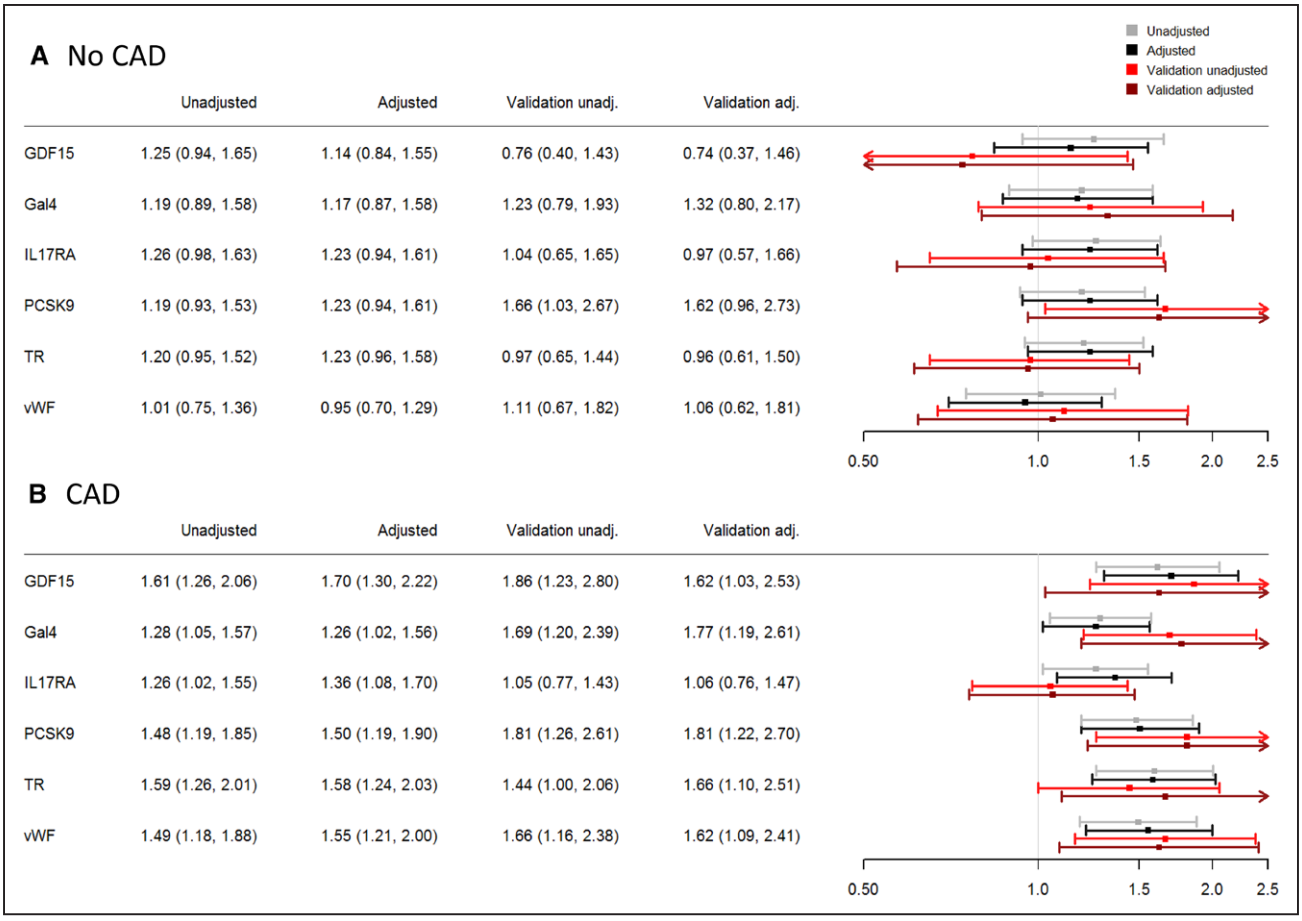

Figure 2. Associations between future surgery for aortic stenosis (AS) and 6 plasma proteins after excluding individuals who provided the blood sample <5 years before surgery.

A, Associations with AS in the absence of concomitant coronary artery disease (CAD). **B**, Associations with AS in the presence of concomitant CAD. Unadjusted and adjusted estimates are shown in gray and black for the discovery cohort and in red and brown for the validation cohort. GDF-15 indicates growth differentiation factor 15; Gal4, galectin-4; IL17RA, interleukin 17 receptor A; PCSK9, proprotein convertase subtilisin/kexin type 9; TR, transferrin receptor protein 1; and vWF, von Willebrand factor.

Galectin-4 is member of the β-galactoside–binding family of proteins. It is found intracellularly and on the cell surface in the intestine but also in the circulation. Intracellular galectin-4 regulates cell proliferation and apoptosis; the extracellular form mediates intercellular adhesion.[24] No previous study has reported an association between circulating levels of galectin-4 and AS. Another member of the galectin family, galectin-3, was shown to be overexpressed in stenotic aortic valves,[25] and it was also identified as a critical participant in the pathogenesis and progression of cardiac fibrosis and inflammation. Moreover, galectin-3 was predictive of clinical outcomes after replacement of a stenotic aortic valve.[26]

AS is a slowly progressive disease. It is often diagnosed late, in connection with the development of symptoms, after stenosis has become severe. However, left ventricle hypertrophy associated with myofibrillar hypertrophy or fibrosis can occur early in the natural history of AS.[27] Thus, some individuals in the present study could have harbored AS in a subclinical, undiagnosed stage at the time of blood sampling. Consequently, they may have already developed extensive valvular and myocardial changes. Therefore, we conducted an analysis that only included individuals who had provided blood samples >5 years before surgery. In that analysis, we found that the associations between AS and GDF-15 and galectin-4 were attenuated and nonsignificant in the absence of concomitant CAD. Furthermore, no evidence of association with non-CAD AS was found in the validation cohort. Patients in the validation cohort were older at surgery, the interval from survey to surgery was longer, and the risk profile differed somewhat compared with the discovery cohort, with more obesity and more frequent glucose intolerance. Even if the number of well-characterized patients is high and comparable to that in similar studies,[28] the lack of association in the validation cohort may be the effect of limited power or the play of chance.

In all, our results suggest that the changes in GDF-15 and galectin-4 might occur, if at all, relatively late in the development of AS. If so, the elevated levels of GDF-15 and galectin-4 would likely be a response

to the stenotic valve rather than part of the causation. In tissue preparations, elevated expressions of galectin-3 and soluble ST2 proteins were previously shown to be associated with established AS.[29,30] In this study, however, we did not find those associations in plasma samples.

The strengths of this study included the strict sample and baseline data collection procedures in the population-based studies, the near-complete information on baseline risk factors, the extensive follow-up information in hospital records, and the inclusion of a validation cohort. Several limitations of this study should also be acknowledged.

First, the features of the study population may have limited the generalizability of our findings. The population had a white background rooted in the northern part of Sweden, which might limit the generalizability of our findings to other populations. The inclusion criteria for the VIP survey may have affected the age distribution of the patient population; that population only included individuals who had participated in the health survey at 30, 40, 50, or 60 years of age. This may have led to an underrepresentation of younger patients. Moreover, the study design did not permit a detailed description of the underlying valve morphology (eg, a bicuspid versus a tricuspid aortic valve). Finally, this study only included patients who met indications for surgery. Thus, we excluded patients with severe comorbidities who represented contraindications for surgery and patients without AVR because of only slightly affected aortic valves. Relatedly, no patients with CAD without AS were included, and our findings could possibly relate to CAD only. However, using PEA in patients with carotid atherosclerosis, 7 proteins were found associated with case status, and only GDF-15 was overlapping with this study.[31]

A second limitation concerned methodology. The subset of patients without CAD was defined by the absence of any visible atherosclerosis, but we could not exclude any atherosclerotic changes in the vessel wall that might not have affected the vessel lumen. Furthermore, the presence of CAD was analyzed as a categorical value (yes/no) without taking into account the extent of atherosclerosis. In addition, the discovery potential was limited by the relatively small number of proteins measured.

The multiplex PEA technology is a high-throughput assay with high specificity and sensitivity for the detection of protein biomarkers in a low sample volume. Moreover, with this method, there is no cross-reactivity during the multiplexing.[12] Recently, a foundational study to compare PEA and conventional immunoassays was performed.[32] More than 9000 samples from 3 large studies in cardiovascular diseases were analyzed for GDF-15 and N-terminal pro B-type natriuretic peptide with commercial kits from Roche Diagnostics on a Cobas e601 analyzer (Roche Diagnostics) and with high-sensitivity ELISA for interleukin-6, and the same samples were also analyzed with a number of PEA panels. Very good correlations were found for the conventional assays and PEA. The results from this study indicated that the PEA technology reliably reflects the levels of all simultaneously measured biomarkers. The PEA also provided the same association to outcome, cardiovascular death, as the gold standard conventional assays. These results indicate that the PEA technique is a suitable tool for biomarker screening purposes. However, the PEA method is only semiquantitative; absolute quantification would require the addition of standards.

The history of the samples also introduced some limitations. At the time that blood samples were drawn, the baseline clinical examination did not include echocardiography. Therefore, we could not assess the presence of calcifications in the aortic leaflets or subclinical AS. Consequently, it was not possible to determine what stage of the disease process might be linked to the discovered protein alterations. Moreover, no data on the presence of peripheral vascular disease were available. Because AS has been shown to be associated with peripheral vascular disease, this omission could have introduced confounding. However, none of the 5 proteins identified has been associated with peripheral vascular disease.[33]

CONCLUSIONS

We provided evidence that alterations in 5 proteins—GDF-15, galectin-4, PCSK9, TR, and vWF—occurred ≈11 years before AS developed to the point of requiring valvular replacement. Moreover, there was some evidence of GDF-15 and galectin-4 being associated with the recent development of nonatherosclerotic AS. It remains uncertain whether these protein alterations were predictive of or responsive to AS development. These results require further validation in external studies.

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Disclosures

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