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ORIGINAL RESEARCH ARTICLE

Comparative serum proteomic profiling of cardiovascular death versus coronary atherosclerosis

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Abstract

Introduction: The proteomic differences distinguishing active vascular inflammation (severe atherosclerosis) from terminal systemic collapse (cardiovascular death [CVD]) remain undefined.

Objective: We aimed to identify protein profiles associated with key functional differences between severe atherosclerosis and CVD.

Methods: Serum proteomic profiles were evaluated in four groups from two cohorts. In the general population, Cohort 1, CVD patients (Group 1, $n = 10$) were compared with healthy controls (Group 2, $n = 10$). In Cohort 2 (coronary heart disease patients), those with severe coronary stenosis (Group 3, $n = 9$) were compared with those without stenosis (Group 4, $n = 9$). Protein expression was analyzed using antibody microarrays targeting 656 proteins across 13 signaling pathways. Findings were validated by in-house enzyme-linked immunosorbent assay for adhesion-regulating molecule 1 (ADRM1) in independent Cohort 3 and total Cohort 1.

Results: Severe atherosclerosis (Group 3 versus Group 4) showed increased levels of inflammation-promoting cytokines (interleukin [IL]-6, IL-1α, tumor necrosis factor-alpha), T-cell mediators (CD53, lymphocyte-specific protein tyrosine kinase, granzyme B), apoptotic regulators, and matrix remodeling proteins (collagen II), indicating active inflammation. In contrast, CVD samples (Group 1 versus Group 2) exhibited lower levels of protective proteins, including vinculin, laminin B1, dysferlin, and calcium signaling mediators. Higher ADRM1 levels were observed in CVD samples, confirmed in Cohort 1 but not in atherosclerosis or nonfatal acute myocardial infarction in Cohort 3. The association between high ADRM1 and CVD was confirmed by Kaplan–Meier survival analysis.

Conclusion: High ADRM1 may be considered an independent predictor of CVD during 6.5-year follow-up. Proteomic profiling suggests that CVD, but not an extension of atherosclerotic burden, may be associated with excessive proteasomal degradation via ADRM1.

Keywords: Proteomics; Atherosclerosis; Cardiovascular death; Adhesion-regulating molecule 1 protein; Apoptosis; Proteostasis

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1. Introduction

Globally, cardiovascular disease continues to be the primary cause of death, with coronary atherosclerosis as the primary underlying pathology, according to the World Health Organization (WHO).[1] Substantial progress has been made in identifying risk factors and biomarkers for atherosclerotic plaque development. However, molecular mechanisms that distinguish stable severe atherosclerosis from terminal cardiovascular death (CVD) events remain poorly understood.[2,3] The majority of proteomic studies have focused on predicting the incidence of CVD or attempting to identify individual protein biomarkers associated with atherosclerosis progression rather than comparing proteome profiles in samples of patients with CVD incidents during a follow-up period with samples from patients with earlier stages of the disease.[4,5] The transition from severe coronary atherosclerosis to CVD likely involves fundamental changes in inflammatory signaling, apoptosis regulation, structural integrity, and proteostasis. However, specific protein expression patterns that characterize these two distinct clinical states have not been systematically compared within a single analytical framework. Understanding molecular differences between severe atherosclerosis and CVD during follow-up may help identify novel therapeutic targets to prevent progression to the terminal CVD stage. In the present study, we performed comparative proteomic profiling in serum samples from patients with CVD events during 6.5-year follow-up, using matched healthy individuals as a reference, and also compared patients with severe coronary atherosclerosis with patients who have unobstructed coronary vessels as a reference. We aimed to identify differentially expressed proteins that distinguish CVD patients and patients with severe coronary atherosclerosis from the corresponding reference groups to characterize the principal functional differences in proteomic profiles between severe atherosclerosis and CVD during follow-up.

2. Materials and methods

2.1. Study participants

Cohort 1 was used for case–control analysis in small groups selected from 222 individuals (134 men and 88 women), including 111 cases and 111 controls. Selection criteria for proteomic profiling included accessible endpoint data and availability of matched healthy control samples. Endpoints for Cohort 1 included CVD during 6.5-year follow-up with the median time of 3.1 (1.6; 4.9) years (minimal time of 0.4 year [166 days], and maximal time of 6.5 years) for a total of 48 cases (77% fatal acute myocardial infarction [AMI] and 23% other causes of CVD; 30 men and 18 women) and nonfatal AMI (63 cases; 37 men, 26 women). Cohort 1 was recruited and monitored as described in our previous work within seven regions of the Russian Federation for the ESSE-RF study.[5] CVD was confirmed by official death certificates, and nonfatal AMI was verified using patient medical records and electrocardiography. Control apparently healthy subjects were recruited from the same regions of the Russian Federation and matched by sex to align with the key demographic and biometric characteristics. These control subjects had no CVD or nonfatal AMI records, were alive throughout the entire follow-up, and had no history of cancer, diabetes, ischemic heart disease, arrhythmia, arthritis, or other cardiovascular conditions; additionally, the control subjects were matched in age (within two years of age of the matching case, region, sex, and education level. Optional matching, if available, included alcohol use, smoking status (never, former, current), and body mass index (BMI). The groups for the present study were selected from a larger multicenter population-based cross-sectional survey, and the overall ESSE-RF study design and preliminary findings have been detailed elsewhere.[5-8] The ESSE-RF study began in 2011, continued into 2015–2016, and was endorsed by three ethics boards in the Russian Federation: National Research Center for Preventive Medicine (NRCPM), Federal Almazov North–West Medical Research Centre, and Russian Cardiology Research-and-Production Complex. Compliance with the Declaration of Helsinki and WHO guidelines was maintained throughout all procedures.[6,7] To ensure consistency across all participating medical centers, enrollment inclusion criteria included a glomerular filtration rate (GFR) of at least 60 mL/min, as determined by the Cockcroft–Gault equation based on serum creatinine. Samples of serum and plasma collected at baseline were stored in the Biobank of NRCPM.

Cohort 2 was used to select groups for proteome profiling and included 500 patients (aged 25 to 86 years, both sexes treated at NRCPM in 2011–2012). The patients underwent coronary angiography based on the European Society of Cardiology guidelines[9] via transfemoral Judkins technique. Angiographic data were captured using a GE Innova 4100 IQ system (GE Medical Systems, United States of America [USA]). Stenosis severity and Gensini scores[10] were assessed using Advantage Workstation v4.4 as described by our group previously.[11] Any medications taken before admission were recorded at the time of blood collection.

Exclusion criteria included: myocardial infarction in the prior six months; active acute inflammation; GFR < 60 mL/min/1.73 m^2; poorly controlled type I or II diabetes (glycated hemoglobin [HbA1c] above 7.5%); left ventricular ejection fraction < 40%; malignancy; familial

hypercholesterolemia; blood disorders; or immune/autoimmune diseases.

The study protocol was approved by the NRCPM Independent Ethics Committee (approval no. 07-05/12), in accordance with the Declaration of Helsinki and WHO guidelines. Written informed consent was obtained from all participants.

Cohort 3 samples were obtained from 214 patients over 25 years of age who were admitted to NRCPM in 2018–2019. All patients underwent coronary angiography and Gensini score evaluation as described by our group previously.[11] Coronary lesions of any degree (Gensini score > 0) were present in 138 patients, and 76 patients (35.5%) had no detectable stenosis of coronary vessels (Gensini score = 0).

Exclusion criteria included active cancer, recent myocardial infarction (past 6 months), acute inflammation, GFR < 60 mL/min/1.73 m^2, poorly controlled diabetes (HbA1c > 7.5%), low ejection fraction (< 40%), familial hypercholesterolemia, blood disorders, or autoimmune diseases. Further details are available in our previous works.[11,12] Written informed consent was obtained from all participants; the NRCPM Independent Ethics Committee approved the protocol (approval No. 09–05/19) in line with the Declaration of Helsinki and WHO guidelines.

2.2. Blood sampling

Blood samples were withdrawn from the cubital vein at baseline. Serum and citrate plasma were obtained by centrifugation at 1,000 g for 15 min at 4 °C, aliquoted, and stored at −26 °C.

2.3. Proteome profiling

2.3.1. Groups for proteome profiling

From Cohort 1, 10 serum samples from patients with CVD (selected from 48 cases) were assigned to Group 1, and 10 matched serum samples from apparently healthy controls were assigned to Group 2 for protein microarray analysis. Endpoints included CVD (median time of 2.6 years [1.5; 5.3]; minimal time of 0.8 years [299 days], and maximal time of 6 years). From Cohort 2, a total of 18 samples were used for proteomic profiling, including nine from patients with severe coronary stenosis (Group 3) and nine from patients with Gensini score = 0 (Group 4). Statistical power calculations for the sample sizes of Groups 1–4 were published previously by our group.[13,14] These calculations demonstrated that at least three microarray sets may be sufficient for prospective evaluation of the target proteins.[13] Top candidate proteins were selected based on comparison of the profiles, and the results were tested by an independent method of in-house sandwich enzyme-linked immunosorbent assay (ELISA) in all samples of Cohort 1 and Cohort 3.

2.3.2. Proteome profiling using antibody microarray

Serum samples were diluted in phosphate-buffered saline (PBS; Arrayit Corporation, USA) to reach 1 mg/mL and then labeled following the manufacturer's protocol (1 µL of Green 540 reagent; Arrayit Corporation, USA). Antibody microarrays (ASB600, Full Moon Biosystems, USA) containing 656 antibodies, each spotted in duplicate, were used for protein profiling. All proteins included in the microarray ASB600 are listed at https://www.fullmoonbio.com/product/explorer-antibody-array/#documentation.

Each slide was loaded with 500 ng of labeled proteins and blocked for 1 h with a solution containing 3% non-fat dry milk (Full Moon Biosystems, USA). After washing, the arrays were incubated for an additional 2 h with a coupling buffer also containing 3% dry milk. Following additional washes, the slides were dried by centrifugation. Scanning was performed with an InnoScan 900 laser scanner (Innopsys, France) under the following settings: normal scan mode, 20 L/s velocity, laser power at 5.0, photomultiplier gain at 100, 10 µm pixel size, and 532 nm wavelength. GAL files and grid templates were loaded automatically and aligned with positive controls. Image analysis was carried out using Mapix software version 7.0.0 (Innopsys, France), and labeled proteins were identified based on microarray-specific GAL files as described by our group previously.[13,15]

2.3.3. In-house enzyme-linked immunosorbent assay of serum ADRM1 (proteasomal adaptor protein)

The levels of adhesion-regulating molecule 1 (ADRM1) were analyzed in serum samples from Cohort 1 and Cohort 3 to validate the results obtained by comparative protein profiling. Samples were diluted to 10 mg/mL with carbonate immobilization buffer (Na_2CO_3 1.5 g, $NaHCO_3$ 2.93 g, distilled water 1 L; Sigma-Aldrich, USA) and added to the wells of a 96-well plate (Jet Biofil, China). After blocking with 1% bovine serum albumin solution (Sigma-Aldrich, USA) in PBS and washing, a rabbit recombinant monoclonal anti-ADRM1 antibody (clone ARM-1; Abcam ab157218, Abcam, United Kingdom) was added at a dilution of 1:10,000 and incubated for 1 h at 37 °C. After washing, the plates were incubated with secondary anti-rabbit antibodies conjugated to horseradish peroxidase (1:10,000; FNSA-0004; Fine Test, China) for 1 h at 37 °C. After adding the peroxidase substrate and color development, the reaction was stopped, and optical density (OD) was read at 450 nm (reference wavelength 620 nm) on a Tecan Infinite 200Pro plate reader (Tecan Group Ltd.,

Switzerland). The results were expressed as normalized units (%) calculated as follows: (sample OD_{450}/mean OD_{450} of all microplate samples) × 100. This procedure standardizes the data for relative comparisons of the ADRM1 concentrations between samples for a coefficient of variation (CV) < 10%.

2.4. Statistical analysis

The fluorescence spots on the microarray images were digitized using Mapix software provided by Innopsys (France) and expressed as median pixels minus background. All values were normalized (%) to the positive controls for each slide as described in our previous work.[12] Parallel spots with a CV exceeding 25% were omitted from all subsequent analysis. The profiles were compared using the mean of normalized spots for each protein, with a CV threshold below 25%. Normalized spots with a CV that deviated from the background area by less than 10% were classified as being similar to the background and were excluded from evaluation.[13]

To resolve the multiple-comparison problem, we used the Holm–Bonferroni correction with a threshold of 0.05. Protein ratios below 1.6 for the CVD profile (Group 1) and below 3 for the severe atherosclerosis profile (Group 3) were excluded from subsequent analysis, as defined previously.[13]

Statistical evaluations were carried out using Statistica version 8.0 (StatSoft, Inc., USA) and SPSS IBM Statistics version 23 (IBM Corp., USA). R-based tools were used for volcano plot visualization. Normality of the data distributions was examined using the Kolmogorov–Smirnov criterion. The data are expressed as the mean and standard deviation or median (Q1; Q3). Two-tailed non-parametric analysis of variance, including Mann–Whitney test with Holm–Bonferroni correction, Welch's *t*-test, and Brown–Forsythe test, was used for group comparisons. Fold change (FC) as $\log_2$FC was calculated as $\log_2$ (control mean/CVD mean) using normalized pixels. Positive values indicate lower abundance in CVD cases. Cohen's *d*–standardized effect size (Cohen's *d*) was used to classify the magnitude of the effect. Absolute values of approximately 0.2, 0.5, and 0.8 are generally considered small, medium, and large effects, respectively. *p*-values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) procedure. FDR < 0.05 was considered statistically significant. Kaplan–Meier and Cox regression analysis was validated with the Wald test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Proteomic profiling using antibody-based microarray

Proteomic profiling of 20 serum samples from Cohort 1 (Groups 1 and 2) and 18 samples of Cohort 2 (Groups 3 and 4) was performed as the ratio of mean values of healthy controls (n = 10) to CVD cases (n = 10) and as the ratio of mean values for severe atherosclerosis (n = 9) versus no coronary stenosis (n = 9), respectively. The groups selected for proteome profiling were comparable in sex, age, BMI, and several biochemical parameters (Tables 1 and 2, respectively). The comparison between these two profiles indicates that the present study can be considered a cross-cohort study.

The differences in the proteomic profiles detected by antibody-based microarray between CVD (Group 1) and matched healthy control (Group 2) were very modest, with a maximum of 3-fold (Tables 3 and S1–S2, Figures S1–S3). However, in patients with atherosclerosis (Group 3), the maximum differences with patients without coronary lesions (Group 4) were 21-fold. The threshold cut-off values were chosen for profile analysis to provide sufficient confidence levels for validation: 3-fold for the atherosclerosis profile (Groups 3 and 4) and 1.5-fold for the CVD profile (Groups 1 and 2) (Tables 4 and S3–S5, Figures S4–S6). Quantitative proteomic profiling revealed a distinct molecular signature associated with CVD during follow-up (Table 3, Figure 1). In contrast to severe atherosclerosis profile (Table 4, Figure 1) characterized by increased levels of proteins usually associated with repair and inflammation signaling, the CVD profile (Table 3) was defined predominantly by decreased levels of proteins involved in structural integrity, calcium signaling, inflammation, apoptosis regulation, and growth factor signaling, accompanied by a standing out increase in a proteasomal adaptor protein (ADRM1) (Figure 1).

Several proteins critical for cellular and extracellular architecture were decreased in the CVD profile, such as vinculin,[16] a key component of focal adhesions and mechanotransduction, and laminin B1, a major noncollagenous constituent of basement membranes.[17] Additionally, dysferlin, a membrane repair protein,[18] showed decreased levels. Calcium-dependent signaling may be markedly affected in the CVD profile because calmodulin,[19] a universal calcium sensor and transducer, and S100 protein, which mediates calcium-dependent inflammatory responses,[20] were decreased. Multiple

Table 1. Baseline characteristics of the groups of the participants selected for microarray analysis from Cohort 1

Parameters	Group 1: CVD (n = 10) (mean ± SD or median [Q1; Q3])	Group 2: Healthy control (n = 10) (mean ± SD or median [Q1; Q3])	p-value[a]
Age, years	59.00 (50.00; 63.00)	59.00 (50.00; 63.00)	0.81
Sex (men, %)	50%	50%	–
Smoking (n): never/former/present	5/0/5	7/2/1	0.08[b]
Diabetes mellitus type 2 (n)	1	0	–
BMI	29.08 ± 3.55	28.03 ± 7.12	0.73
Total cholesterol, mmol/L	5.35 ± 1.10	5.28 ± 1.30	0.81
LDL-cholesterol, mmol/L	3.17 ± 1.08	3.86 ± 0.95	0.21
HDL-cholesterol, mmol/L	1.28 ± 0.38	1.55 ± 0.25	0.06
Lipoprotein a, mg/dL	11.80 (2.00; 27.50)	8.70 (6.90; 90.00)	0.59
Creatinine, μmol/L	71.69 ± 7.59	71.01 ± 4.77	0.97
GFR, mL/min/1.73 m^2	111.46 ± 34.94	109 ± 28.81	0.76
CVD time, days	1200.60 ± 743.24	–	–
Medication (n)	None	None	–

Notes: [a]p-value calculated by Mann–Whitney U test for all variables. [b]Pearson's chi-squared test with Yates' correction was used to calculate p-value for 2 × 2 chi-square tests.
Abbreviations: BMI: Body mass index; CVD: Cardiovascular death; GFR: Glomerular filtration rate according to Cockcroft–Gault formula; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; SD: Standard deviation.

Table 2. Baseline characteristics of the groups of patients selected for microarray analysis from Cohort 2

Parameters	Group 3 Gensini score > 1 (n = 9) (mean ± SD or median [Q1; Q3])	Group 4 Gensini score = 0 (n = 9) (mean ± SD or median [Q1; Q3])	p-value[a]
Gensini score	60.00 [31.75; 73.75]	0.00 [0.00; 0.00]	–
Age, years	56.00 ± 10.34	60.00 ± 10.94	0.33
Sex (n), men	5	5	>0.05[b]
Smoking (n): never/former/present	5/3/1	7/0/1	0.06[b]
BMI	30.38 ± 5.87	27.17 ± 4.75	0.28
Diabetes mellitus type 2 (n), no/yes	6/3	8/1	0.57[b]
Total cholesterol, mmol/L	5.47 ± 1.53	5.32 ± 1.31	0.96
LDL-cholesterol, mmol/L	3.77 ± 1.30	3.39 ± 1.26	0.75
HDL-cholesterol, mmol/L	1.04 ± 0.39	1.05 ± 0.16	0.70
Lipoprotein a, mg/dL	10.00 [3.15; 34.15]	15.50 [8.07; 24.55]	0.48
Creatinine, μmol/L	86.67 ± 13.90	88.89 ± 12.77	0.69
Medications[c]			
Statins (n; no/yes)	5/4	7/2	0.62[b]
Statins, dose (mg)	50.0 ± 26.7	26.6 ± 19.4	0.69
Antihypertensive drugs (n; no/yes)	8/1	9/0	0.05[b]

Notes: [a]p-value calculated by Mann–Whitney U test for all variables. [b]Pearson's chi-squared test with Yates' correction was used to calculate p-value for 2 × 2 chi-square tests. [c]Medications before baseline: 6 months to 1 day prior to blood withdrawal.
Abbreviations: BMI: Body mass index; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; SD: Standard deviation.

Table 3. Comparative proteomic profiling of 20 serum samples from Cohort 1 was performed as the ratio between healthy controls (Group 2; *n* = 10) and CVD cases (Group 1; *n* = 10)

ID-ASB600	Name in ASB600	Ratio: Healthy control/CVD (normalized pixels[a])	*p*[b]	Role in signaling	General effects
Downregulation in CVD					
397	Vinculin	1.46	0.037	Protein of the adhesion complex that contributes to the organization of an entire signaling network	Cytoskeleton/focal adhesion
133	S100	1.76	0.010	Inflammation/innate immunity; alarmin that promotes neutrophil recruitment	Inflammation/innate immunity
576	Neutrophil elastase	1.81	0.035	Inflammation/ECM remodeling, neutrophil activation and tissue degradation	Inflammation/ECM remodeling
593	Dysferlin	1.84	0.030	Membrane repair/muscle; sarcolemma repair, Ca^{2+}-dependent vesicle fusion	Inflammation/ECM remodeling
415	Calmodulin	1.90	0.014	Calcium signaling; Ca^{2+} sensor, regulates protein kinases, contraction, apoptosis	Membrane repair/calcium signaling
609	CD23	1.98	0.038	Immune regulation/B-cell activation; IgE receptor, promotes inflammation	Calcium signaling/ apoptosis
122	E2F-3	2.27	0.038	Cell cycle/proliferation; transcription factor, G1/S transition	Inflammation/B-cell immunity
310	Laminin B1/b1	2.27	0.036	ECM/basement membrane; cell adhesion, migration, vascular integrity	Cell cycle/proliferation
494	TRADD	2.30	0.024	Apoptosis/TNF signaling; adaptor protein for TNFR1, mediates NF-κB and cell death	Extracellular matrix/adhesion and pro-apoptotic (TNF receptor signaling
540	FLIP	2.35	0.017	Apoptosis inhibition; blocks caspase-8 activation, promotes survival	Apoptosis/TNF signaling
468	DP-2	2.36	0.037	Cell cycle/transcription; dimerization partner for E2F	Apoptosis (anti-apoptotic)
139	Caspase 6 (Mch2)	2.37	0.037	Apoptosis execution; cleaves laminins, involved in neuronal death	Cell cycle/transcription
391	G-CSF	2.39	0.032	Hematopoiesis/inflammation; neutrophil production and mobilization	Apoptosis execution
19	Gab-1	2.96	0.012	Growth factor signaling; adaptor for PI3K/AKT downstream of receptor tyrosine kinases	Hematopoiesis/inflammation
220	mGluR1	3.20	0.028	Neurotransmitter signaling; metabotropic glutamate receptor; also expressed in the heart	Growth factor/PI3K-AKT signaling
Upregulation in CVD					
61	ADRM1	−3	0.032	Ubiquitin–proteasome system; proteasomal ubiquitin receptor	Ubiquitin–proteasome system

Notes: [a]The spots on the images were quantified as median pixel values minus background and normalized (%) to the positive controls for each slide. [b]*p*: Mann–Whitney U test with Holm–Bonferroni correction.

Abbreviations: ADRM1: Adhesion regulating molecule 1 (also known as hRpn13, a proteasomal ubiquitin receptor); CVD: Cardiovascular death; CD23: Low-affinity immunoglobulin epsilon Fc receptor; DP-2; Differentiation plasmid 2 (often refers to transcription factor DP-2, a dimerization partner for E2F; also known as TFDP2); E2F-3: E2F transcription factor 3; ECM: Extracellular matrix; FLIP: FLICE (FADD-like IL-1β-converting enzyme)-like inhibitory protein (also known as CFLAR); Gab-1: GRB2-associated-binding protein 1; G-CSF: Granulocyte colony-stimulating factor; mGluR1: Metabotropic glutamate receptor 1; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; TNF: Tumor necrosis factor; TNFR1: Tumor necrosis factor receptor 1; TRADD: Tumor necrosis factor receptor type 1-associated death domain protein.

Table 4. Comparative proteomic profiling of 18 serum samples from Cohort 2 was performed as the ratio between patients with severe coronary lesions (Group 3; *n* = 9) and those with an apparent lack of coronary lesions (Group 4; *n* = 9)

ID ASB600	Name in ASB600	Ratio: severe coronary lesions/lack of coronary lesions (normalized pixels[a])	*p*[b]	Role in signaling
567	Cadherin-P	21.08	0.001	Cell adhesion; involved in cadherin/catenin signaling and gamma-secretase pathway
304	Ang-2	18.72	0.018	Angiopoietin/Tie signaling; promotes angiogenesis and inflammation
570	TRAP	18.44	0.001	Mammalian TAR DNA-binding protein
487	IL-5	12.77	0.003	JAK-STAT pathway (via beta-c receptor); regulates eosinophil production
162	IL-1 alpha	12.33	0.030	NF-κB and MAP kinase pathways; key mediator of innate immunity and inflammation
46	FGF-2	9.94	0.024	Receptor tyrosine kinases and the MAPK/ERK and PI3K-AKT pathways; regulates angiogenesis and cell proliferation
28	FGF-1	6.45	0.035	Receptor tyrosine kinases (FGFR) signaling; regulates cell growth, differentiation, and survival
391	G-CSF	4.89	0.020	JAK-STAT (primarily STAT3), Ras/MAPK, and PI3K/AKT pathways; regulates granulopoiesis
17	GSTmu	4.81	0.002	Metabolism/detoxification (glutathione S-transferase); regulated by the Ras pathway
555	Nitric oxide synthase, brain (bNOS)	4.61	0.001	Nitric oxide signaling; produces nitric oxide for synaptic plasticity and vasodilation
177	DFF45/ICAD	4.40	0.004	Apoptosis (caspase-activated DNase regulation); inhibitor of caspase-activated DNase
103	Collagen II	3.93	0.005	ECM–receptor interaction; structural component of cartilage
114	IL-6	3.92	0.010	JAK-STAT (STAT3), MAPK, and NF-κB pathways; regulates inflammation and immune response
625	FHIT	3.89	0.003	Tumor suppression; involves apoptosis and cell cycle regulation
528	Lck (p56lck)	3.88	0.002	T cell receptor signaling (Src family kinase); critical for T cell activation and differentiation
538	I-Kappa-B kinase b (IKKb)	3.74	0.004	NF-κB pathway; phosphorylates IκB, leading to NF-κB activation
167	TNFa	3.66	0.002	NF-κB and apoptosis (caspase) pathways; regulates inflammation and cell death
499	MMP-16/MT3-MMP	3.66	0.003	ECM remodeling; activates pro-MMP2 (gelatinase A) involved in metastasis
516	Caspase 1	3.60	0.003	Inflammasome pathway; cleaves pro-IL-1β and pro-IL-18 into active cytokines
81	Inhibin alpha	3.32	0.001	TGF-beta superfamily signaling; inhibits activin, regulating the pituitary–gonadal axis
147	GM-CSF	3.26	0.014	JAK-STAT (via beta-c receptor) and PI3K pathways; stimulates stem cell production
606	Myoglobin	3.20	0.027	Oxygen transport; not a signaling protein, but a hemoprotein for oxygen storage in muscle
566	MyD88	3.16	0.009	TLR/IL-1 signaling (adapter protein); activates NF-κB and MAPK pathways

(*cont'd...*)

Table 4. (*Countinued*)

ID ASB600	Name in ASB600	Ratio: severe coronary lesions/lack of coronary lesions (normalized pixels[a])	*p*[b]	Role in signaling
513	Granzyme B	3.14	0.002	Apoptosis (cytotoxic); cleaves caspases and Bid to induce target cell death
655	Oct-3	3.14	0.007	Stem cell pluripotency; transcription factor regulating self-renewal
224	Cadherin-E	3.13	0.008	Cell adhesion/Wnt signaling; sequesters beta-catenin to regulate gene expression
604	GluR6/7	3.13	0.003	Glutamatergic signaling (ion channel); mediates excitatory synaptic transmission
508	p73	3.04	0.006	Apoptosis and development (p53 family); regulates cell cycle and DNA damage response
427	CD3zeta	3.03	0.001	T cell receptor signaling (ITAM domains); transduces activation signals via ZAP70
643	P504S	3.03	0.000	Metabolism; diagnostic marker for prostate cancer, involved in fatty acid metabolism
545	SODD	3.01	0.005	TNF signaling (silencer of death domain); inhibits TNFR1 signaling until ligand binds
593	Dysferlin	3.00	0.005	Membrane repair; involved in vesicle fusion in muscle cells
411	Ferritin	3.00	0.007	Iron homeostasis; sequesters intracellular iron
159	CA19-9	2.99	0.001	Cell adhesion glycoprotein; cancer marker involved in metastasis
132	Bcl-6	2.99	0.016	Transcription factor; represses inflammation and regulates B-cell development
611	GluR1	2.99	0.003	Glutamatergic signaling (AMPA receptor); mediates fast synaptic transmission & plasticity
507	Cullin-3 (CUL-3)	2.94	0.002	Ubiquitin–proteasome (E3 ligase); targets specific proteins for degradation
550	Caspase 8 (FLICE)	2.93	0.002	Apoptosis extrinsic pathway; initiator of caspases activated by death receptors
623	HDAC1	2.91	0.002	Epigenetics; deacetylates histones to regulate chromatin structure and gene expression.
188	Ornithine decarboxylase	2.91	0.002	Metabolism/mTOR (polyamine synthesis); rate-limiting enzyme for cell proliferation
527	NF kappa B/p50	2.90	0.002	NF-κB pathway (transcription factor); dimerizes with p65 to regulate immune genes
362	Bonzo/STRL33/ TYMSTR	2.90	0.002	Chemokine signaling (G-protein coupled receptor); co-receptor for human immunodeficiency virus/simian immunodeficiency virus

Notes: Table 4 contains the top 5.9% (34) of statistically significant proteins from the total dataset with the largest effect sizes. Percentage of FDR-significant proteins retained after applying fold-change thresholds (See Supplement File). [a]See the legend to Table 3. [b]*p*: Mann–Whitney U test with Holm–Bonferroni correction. Abbreviations: AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Ang-2: Angiopoietin-2; Bcl-6: B-cell lymphoma 6 protein; Bonzo/STRL33/TYMSTR: C-X-C motif chemokine receptor 6 (CXCR6); CA19-9: Carbohydrate antigen 19-9 (also sialyl Lewis[a]; a tumor marker); CD3zeta: Cluster of differentiation 3 zeta chain (CD247, T-cell receptor zeta chain); DFF45/ICAD: DNA fragmentation factor 45/inhibitor of caspase-activated DNase; ECM: Extracellular matrix; FGF-1: Fibroblast growth factor 1 (also known as aFGF); FGF-2: Fibroblast growth factor 2 (also known as bFGF); FGFR: Fibroblast growth factor receptor; FHIT: Fragile histidine triad protein; G-CSF: Granulocyte colony-stimulating factor; GluR1: Glutamate receptor 1 (AMPA-type ionotropic receptor; gene *GRIA1*); GluR6/7: Glutamate receptor 6/7 (ionotropic kainate receptor subunits; gene names *GRIK2* and *GRIK3*); GM-CSF: Granulocyte-macrophage colony-stimulating factor; GSTmu: Glutathione S-transferase mu (class Mu.); HDAC1: Histone deacetylase 1; IL: Interleukin; IκB: inhibitor of kappa B; ITAM: Immunoreceptor tyrosine-based activation motif; Lck (p56lck): Lymphocyte-specific protein tyrosine kinase (56 kDa isoform); MMP2: Matrix metalloproteinase-2; MMP-16/MT3-MMP: Matrix metalloproteinase 16/membrane-type 3 MMP; mTOR: Mechanistic target of rapamycin; MyD88: Myeloid differentiation primary response 88; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; Oct-3: Octamer-binding transcription factor 3 (also known as POU5F1, often confused with Oct-4 in humans; in mouse, Oct-3 is Pou5f1); P504S: Protein 504S (also known as AMACR: alpha-methylacyl-CoA racemase); p65: Nuclear factor NF-κB p65 subunit; p73: Protein 73 (tumor suppressor, p53 family member; gene *TP73*); TAR: Trans-activation response element; TGF: Transforming growth factor; TLR: Toll-like receptor; TNF: Tumor necrosis factor; TNFa: Tumor necrosis factor alpha (standard symbol: TNF-α); TNFR1: Tumor necrosis factor receptor 1; TRAP: TNF receptor-associated periodic syndrome protein (or in some contexts tartrate-resistant acid phosphatase; also known as ACP5); ZAP70: Zeta-chain-associated protein kinase 70.

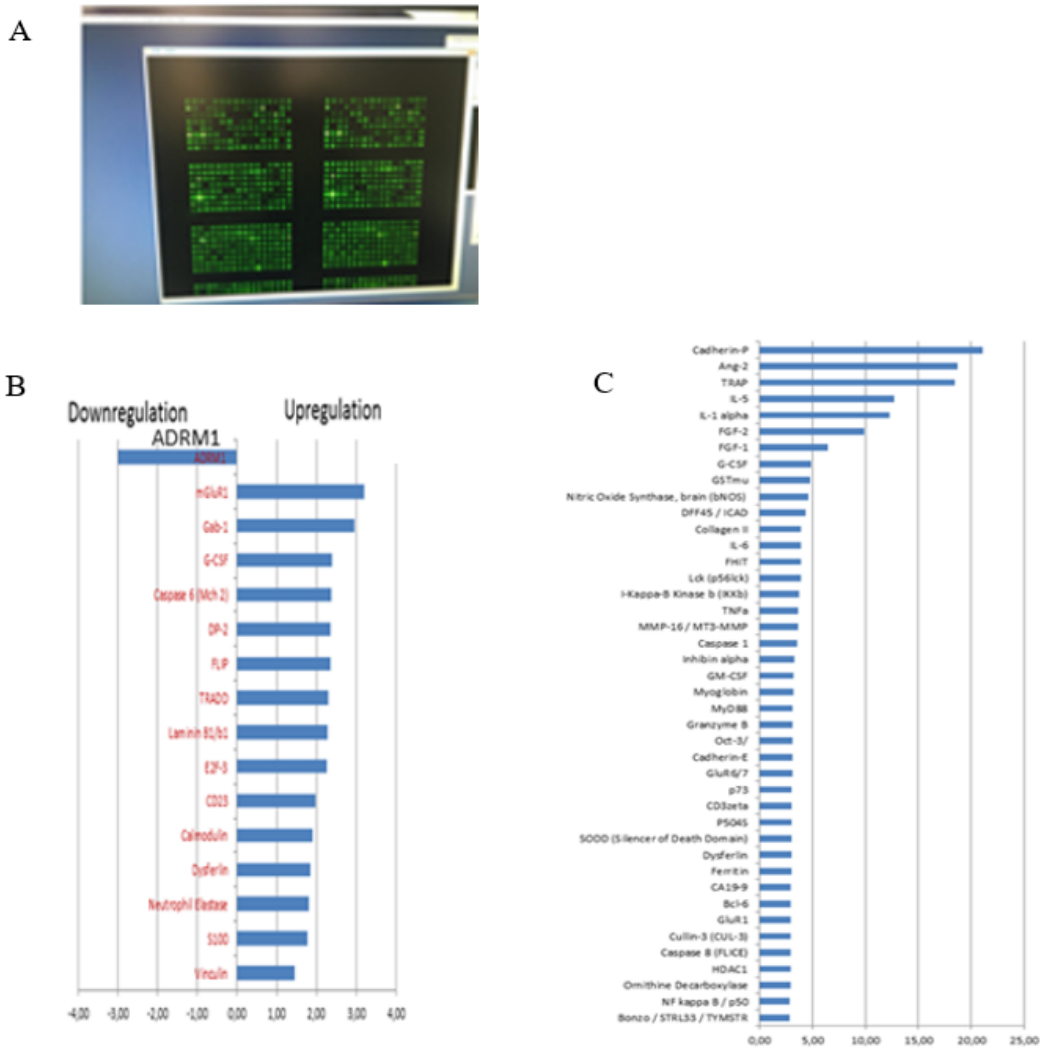

Figure 1. Comparison of serum protein profiles in individuals with cardiovascular death versus matched healthy controls, and in patients with and without severe coronary atherosclerosis. (A) A representative microarray image illustrating the data. (B) Serum protein levels were compared in 10 Group 1 patients with CVD during follow-up and 10 Group 2 matched healthy controls. Proteins with a CVD/control ratio of normalized pixel intensities below 1.6 were excluded. (C) Serum protein levels were compared in nine Group 3 patients with severe coronary stenosis confirmed by angiography and nine Group 4 matched controls without coronary lesions. Only proteins showing upregulation in severe coronary stenosis were shown, and proteins with a stenosis/control ratio of normalized pixel intensities below 3 were excluded. Note: The proteins are arranged in order of decreasing control/CVD ratio.
Abbreviations: Ang-2: Angiopoietin-2; Bcl-6: B-cell lymphoma 6 protein; Bonzo/STRL33/TYMSTR: C-X-C motif chemokine receptor 6 (CXCR6); CA19-9: Carbohydrate antigen 19-9 (also sialyl Lewis[a]; a tumor marker); CD3zeta: Cluster of differentiation 3 zeta chain (CD247, T-cell receptor zeta chain); CD23: Low-affinity immunoglobulin epsilon Fc receptor; CVD: Cardiovascular death; DFF45/ICAD: DNA fragmentation factor 45/inhibitor of caspase-activated DNase; DP-2; Differentiation plasmid 2 (often refers to transcription factor DP-2, a dimerization partner for E2F; also known as TFDP2); E2F3: E2F transcription factor 3; FGF-1: Fibroblast growth factor 1 (also known as aFGF); FGF-2: Fibroblast growth factor 2 (also known as bFGF); FHIT: Fragile histidine triad protein; FLIP: FLICE (FADD-like IL-1β-converting enzyme)-like inhibitory protein (also known as CFLAR); Gab1: GRB2-associated-binding protein 1; G-CSF: Granulocyte colony-stimulating factor; GluR1: Glutamate receptor 1 (AMPA-type ionotropic receptor; gene *GRIA1*); GluR6/7: Glutamate receptor 6/7 (ionotropic kainate receptor subunits; gene names *GRIK2* and *GRIK3*); GM-CSF: Granulocyte-macrophage colony-stimulating factor; GSTmu: Glutathione S-transferase mu (class Mu.); HDAC1: Histone deacetylase 1; IL: Interleukin; Lck (p56lck): Lymphocyte-specific protein tyrosine kinase (56 kDa isoform); mGluR1: Metabotropic glutamate receptor 1; MMP-16/MT3-MMP: Matrix metalloproteinase 16/membrane-type 3 MMP; MyD88: Myeloid differentiation primary response 88; Oct-3: Octamer-binding transcription factor 3 (also known as POU5F1, often confused with Oct-4 in humans; in mouse, Oct-3 is Pou5f1); P504S: Protein 504S (also known as AMACR: alpha-methylacyl-CoA racemase); p73: Protein 73 (tumor suppressor, p53 family member; gene *TP73*); TNFa: Tumor necrosis factor alpha (standard symbol: TNF-α); TRADD: Tumor necrosis factor receptor type 1-associated death domain protein. TRAP: TNF receptor-associated periodic syndrome protein (or in some contexts tartrate-resistant acid phosphatase; also known as ACP5).

immune-related proteins were also decreased in the CVD profile. Neutrophil elastase, a mediator of acute innate immunity,[21] low-affinity immunoglobulin epsilon Fc receptor (CD23),[22] an IgE receptor implicated in B-cell activation and immune modulation, and granulocyte colony-stimulating factor (G-CSF),[23] which was upregulated in severe atherosclerosis, were decreased in the CVD profile.

The CVD profile (Table 3) has pro-apoptotic features. Tumor necrosis factor receptor type 1-associated death domain protein (TRADD), an adaptor protein for TNF receptor-mediated apoptosis,[24] and FLICE-like inhibitory protein (FLIP),[25] an anti-apoptotic protein that inhibits caspase-8, were decreased. This finding is particularly significant given that severe atherosclerosis was associated with upregulation of another anti-apoptotic protein, silencer of death domain (SODD).[26] Caspase 6 (Mch2), an executioner caspase, was also downregulated in the CVD profile despite being increased in the severe atherosclerosis profile [27] (Table 4).

Multiple components of growth factor and transcriptional networks were decreased in the CVD profile. GRB2-associated-binding protein 1,[28] a scaffolding adaptor for several growth factor receptors (including epidermal growth factor receptor and hepatocyte growth factor receptor) and transcription factor E2F3 (E2F3),[29] a cell cycle transcription factor required for S-phase entry, were decreased. An E2F3 dimerization agent, differentiation plasmid 2 (DP-2), was similarly decreased (Table 3). Finally, metabotropic glutamate receptor 1 (GluR1)[30] was decreased in the CVD profile (Table 3), even though other glutamate receptor subtypes (GluR1 and glutamate receptor 6/7 [GluR6/7])[31] were upregulated in the severe atherosclerosis profile (Table 4).

Overall, the CVD proteomic profile (Table 3) was associated with decreased levels of proteins know to be connected to: (i) structural elasticity and resilience (vinculin, laminin B1, and dysferlin); (ii) inflammatory and immune responses (neutrophil elastase, CD23, and G-CSF); (iii) calcium homeostasis (calmodulin and S100); (iv) apoptosis (downregulation of FLIP, despite reduced TRADD and caspase 6); (v) growth and repair signaling (Gab1, E2F3, and DP-2); and (vi) proteasomal degradation (upregulation of ADRM1; Table 5).

Only a single protein in the CVD death profile showed increased levels compared with healthy controls: ADRM1 was upregulated (adhesion regulating molecule 1, alternatively known as Rpn13),[32] a ubiquitin receptor of the 26S proteasome (Figure 1, Table 3).

In contrast to the CVD follow-up signature (Table 3), quantitative proteomic profiling of severe coronary atherosclerosis (Table 4) revealed a complex molecular signature characterized by widespread increase of proteins involved in inflammation, extracellular matrix remodeling, apoptosis regulation, cell signaling, and transcriptional control. Unlike the CVD profile, severe atherosclerosis was characterized by increased levels of proteins linked to active, pro-inflammatory, and pro-repair signaling with concurrent activation of both pro-apoptotic and anti-apoptotic mechanisms. Multiple proteins involved

Table 5. Summary of principal differences between severe atherosclerosis and CVD[a] profiles

Feature	Severe atherosclerosis	CVD
Overall pattern	Widespread upregulation	Widespread downregulation + single upregulation (ADRM1)
Caspase activity	↑ Active (Caspase 1, 8)	↓ Depleted (Caspase 6 down)
Anti-apoptotic factors	↑ SODD (present)	↓ FLIP (lost)
Membrane repair	↑ Dysferlin	↓ Dysferlin
Myeloid growth factor	↑ G-CSF	↓ G-CSF
Structural integrity	↑ Collagen II (repair)	↓ Vinculin, ↓ Laminin B1 (breakdown)
Proteasome pathway	Not prominent	↑ ADRM1 (excessive degradation)
Immune state	Active inflammation (T-cell, cytokines)	Immune exhaustion/loss of CD23
Outcome	Survival with severe disease	Death

Note: [a]CVD during 6.5-year follow-up.
Abbreviations: ADRM1: Adhesion-regulating molecule 1; CD23: Low-affinity immunoglobulin epsilon Fc receptor; CVD: Cardiovascular death; FLIP: FLICE-like inhibitory protein; G-CSF: Granulocyte colony-stimulating factor; SODD: Silencer of death domain.

in cell–cell adhesion and matrix structure were increased: cadherin-P and cadherin-E[11,12] (epithelial cadherin), collagen II,[33] a major structural element of the extracellular matrix, and dysferlin,[18] a membrane repair protein.

A broad spectrum of inflammatory mediators was elevated in the severe atherosclerosis profile (Table 4, Figure 1): interleukin (IL)-5,[34] IL-1α, and IL-6.[34] Tumor necrosis factor alpha (TNFα)[34,35] was increased, which is the key driver of vascular inflammation and endothelial activation. G-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF), which mediate myeloid cell production and differentiation,[23] were both elevated. Granzyme B,[36] a serine protease released by cytotoxic lymphocytes, and myeloid differentiation primary response 88 (MyD88),[37] an adaptor protein downstream of Toll-like receptors and the IL-1 receptor family, were increased.

Several growth factors were increased in the severe atherosclerosis profile (Table 4): fibroblast growth factor 1 (FGF-1) and fibroblast growth factor 2 (FGF-2)[38] were both elevated. Angiopoietin-2 (Ang-2)[39] and inhibin alpha, a member of the TGF-β superfamily,[40] were also increased.

In the severe atherosclerosis profile (Table 4), both pro-apoptotic and anti-apoptotic proteins were upregulated. Pro-apoptotic proteins included caspase-1 (activates inflammatory cytokines and pyroptosis), caspase-8[41] (initiates extrinsic apoptosis via death receptor pathways), and protein 73 (p73;[42] p53 family member; induces apoptosis or cell cycle arrest). Anti-apoptotic proteins included SODD,[26] which binds TNF receptor 1 to prevent spontaneous death signaling, B-cell lymphoma 6 protein (Bcl-6;[43] transcriptional repressor promoting cell survival and inhibiting apoptosis), FLIP[44] (inhibits pro-apoptotic caspase-8), DNA fragmentation factor 45/inhibitor of caspase-activated DNase,[45] which inhibits caspase-activated DNase, preventing DNA fragmentation, and TNF receptor-associated protein (TNF receptor-associated periodic syndrome protein), which modulates TNF receptor signaling.[46]

Upregulated immune-related proteins in the severe atherosclerosis profile (Table 4) included lymphocyte-specific protein tyrosine kinase (56 kDa isoform) (Lck or p56lck),[47] a Src-family tyrosine kinase that mediates T-cell receptor signaling, Cluster of differentiation 3 zeta chain (CD3zeta or CD247), a T-cell receptor zeta-chain essential for T-cell activation, C-X-C motif chemokine receptor 6 (CXCR6), a chemokine receptor involved in lymphocyte recruitment and inflammation, I-kappa-B kinase beta (IKKβ) that activates NF-κB pathway,[48] and NF-κB p50 subunit[24] that regulates inflammation, immunity, and cell survival. Other upregulated proteins included cullin-3, a ubiquitin ligase component regulating protein turnover;[49] neuronal nitric oxide synthase, producing nitric oxide and regulating vascular tone;[15] octamer-binding transcription factor 3 or POU5F1,[50] a transcription factor regulating pluripotency-associated genes; GluR6/7 and GluR1, ionotropic glutamate receptor subunits mediating glutamate signaling;[51,52] histone deacetylase 1,[52] modulating chromatin structure and gene repression; ornithine decarboxylase (rate-limiting enzyme in polyamine synthesis), regulating cell metabolism and proliferation; fragile histidine triad protein, tumor suppressor involved in apoptosis and cell cycle regulation;[53] glutathione S-transferase mu (GSTmu)[54] involved in detoxification and oxidative stress response; matrix metalloproteinase 16, membrane-type 3 (MMP-16) involved in extracellular matrix remodeling and cell migration; protein 504S (an antibody against alpha-methylacyl-CoA racemase and acts as a peroxisomal and mitochondrial metabolic enzyme);[55] ferritin (involved in iron storage and antioxidant defense); carbohydrate antigen 19-9 (a tumor-associated glycoprotein elevated in inflammation); and myoglobin (oxygen-binding heme protein, possibly indicating muscle injury or metabolic adaptation).

Therefore, the severe coronary atherosclerosis proteomic profile (Table 4) is associated with elevated levels of proteins known to be involved in: (i) inflammation (IL-1α, IL-5, IL-6, TNFα, GM-CSF, G-CSF, MyD88, and granzyme B), (ii) extracellular matrix remodeling (collagen II, cadherin-P, cadherin-E, and MMP-16), (iii) angiogenesis and vascular remodeling (FGF-1, FGF-2, and Ang-2), (iv) complex apoptosis regulation (upregulation of pro-apoptotic caspase-1, caspase-8, and p73 and anti-apoptotic SODD and Bcl-6 proteins), (v) NF-κB signaling (IKKβ and NF-κB p50), (v) immune cell activation (Lck, CD3zeta, and Bonzo/CXCR6, and (vi) metabolic and oxidative stress responses (GSTmu, ferritin, and ornithine decarboxylase).

The principal differences reflected in Table 5 revealed a shift from active repair and inflammation in severe atherosclerosis to loss of structural integrity, immune alteration, and pro-apoptotic signaling in CVD death. Upregulation of ADRM1 in CVD appeared to represent a specific pathway that was used to discriminate the CVD proteomic profile. Thus, ADRM1 was selected for subsequent validation using an in-house developed ELISA in an expanded number of samples, including Cohort 1 and Cohort 3.

3.2. Validation of ADRM1 as a potential specific cardiovascular death marker using enzyme-linked immunosorbent assay

The upregulation of ADRM1 was confirmed using ELISA in

all participants with CVD compared with matched healthy control samples in Cohort 1 (% OD_{450}: 114.7 ± 36.39 versus % OD_{450}: 85.4 ± 35.6, respectively; Table 6 and Figure 2A). The associations between ADRM1 levels and CVD incidence were confirmed using the receiver operating characteristic curve (area under the curve = 0.776; p = 0.0001; Figure 2B). Kaplan–Meier survival analysis demonstrates that higher ADRM1 levels were associated with an increased rate of CVD incidence (Mantel–Cox log-rank test, $p < 0.0001$; Figure 3). As demonstrated by univariate Cox regression, CVD incidence was increased by 3.23-fold in participants with ADRM1 levels higher than the median compared with those with ADRM1 levels less than the median (hazard ratio = 3.23; 95% confidence interval: [1.73–6.23]; $p < 0.001$). According to multivariate Cox regression analysis, ADRM1 levels above the median demonstrate an independent association with CVD after adjustment for various parameters, including creatinine levels, since variability in renal function may influence circulating protein patterns through mechanisms such as low-grade inflammation, oxidative stress, altered proteostasis, and immune modulation (Table 7). ADRM1 levels did not differ between AMI participants and matched healthy controls in Cohort 1 and between patients with coronary stenosis versus patients without coronary stenosis in Cohort 3 (Table 6). Thus, high ADRM1 may be an independent specific predictor of CVD.

4. Discussion

The present study revealed that severe coronary atherosclerosis and CVD during follow-up exhibit substantially distinct proteomic signatures. Severe atherosclerosis is characterized by widespread increases in inflammatory, reparative, and apoptosis-associated proteins, whereas CVD during follow-up is defined predominantly by downregulation of protective factors and isolated upregulation of the pro-degradation protein ADRM1. These findings may suggest that CVD is not a simple extension of the advanced atherosclerotic process. We propose that transition from compensated atherosclerosis to CVD is associated with failure of multiple protective systems rather than worsening of inflammation. The majority of large-scale proteomic studies, such as the UK Biobank proteomics study (n = 50,057), were focused on risk prediction rather than mechanistic comparison of disease stages.[3] The present study contributes to our understanding of the mechanistic events by directly comparing the proteomic profiles in severe atherosclerosis and delayed CVD within the same analytical framework on a limited scale, revealing molecular signatures of compensated versus decompensated cardiovascular disease, respectively.

The proteasomal degradation pathway stands out in this context from other signaling mechanisms. ADRM1

Table 6. Serum ADRM1 levels (enzyme-linked immunosorbent assay) in total Cohort 1 and in Cohort 3

ADRM1	*n*	% OD_{450} (normalized units; mean [SD])	Test	p^a between the groups
Cohort 1				
Healthy control	48	85.4 (35.6)	Welch's *t*-test	0.0001
CVD	48	114.7 (36.39)	Brown–Forsythe test	0.0001
Total	96	100.2 (38.74)		
Healthy control	63	101.2(24.33)	Welch's *t*-test	0.9
AMI	63	100.9 (34.27)	Brown–Forsythe test	0.9
Total	126	101.08(29.56)		
Cohort 3				
Patients with no coronary lesions	76	96.90 (21.3)	Welch's *t*-test	0.4
Patients with severe coronary lesions	138	103.73(33.2)	Brown–Forsythe test	0.4
Total	214	101.4 (31.1)		

Note: [a]p between the groups according to Welch's *t*-test and Brown–Forsythe test.
Abbreviations: ADRM1: Adhesion-regulating molecule 1; AMI: Acute myocardial infarction; CVD: Cardiovascular death; OD: Optical density; SD: Standard deviation.

Table 7. Multivariate Cox regression analysis of ADRM1 levels (% OD normalized units; median cut-off) adjusted for age, sex, and other parameters in participants with CVD from Cohort 1

Variable	Coefficient	Standard error	Wald	*p*	HR	95% CI
Age	−0.00	0.02	0.05	0.81	1.00	0.96–1.04
Sex	−0.35	0.33	1.18	0.27	0.70	0.37–1.33
Diabetes mellitus type 2	1.08	0.46	5.51	0.019	2.96	1.20–7.30
LDL-cholesterol	0.15	0.12	1.48	0.223	1.16	0.91–1.49
GFR	0.001	0.004	0.004	0.95	1.00	0.99–1.01
ADRM1	1.016	0.005	10.45	0.001	2.83	1.47–5.44

Abbreviations: ADRM1: Adhesion-regulating molecule 1; CVD: Cardiovascular death; CI: Confidence interval; HR: Hazard ratio; LDL: Low-density lipoprotein; GFR: Glomerular filtration rate according to the Cockcroft–Gault formula; OD: Optical density.

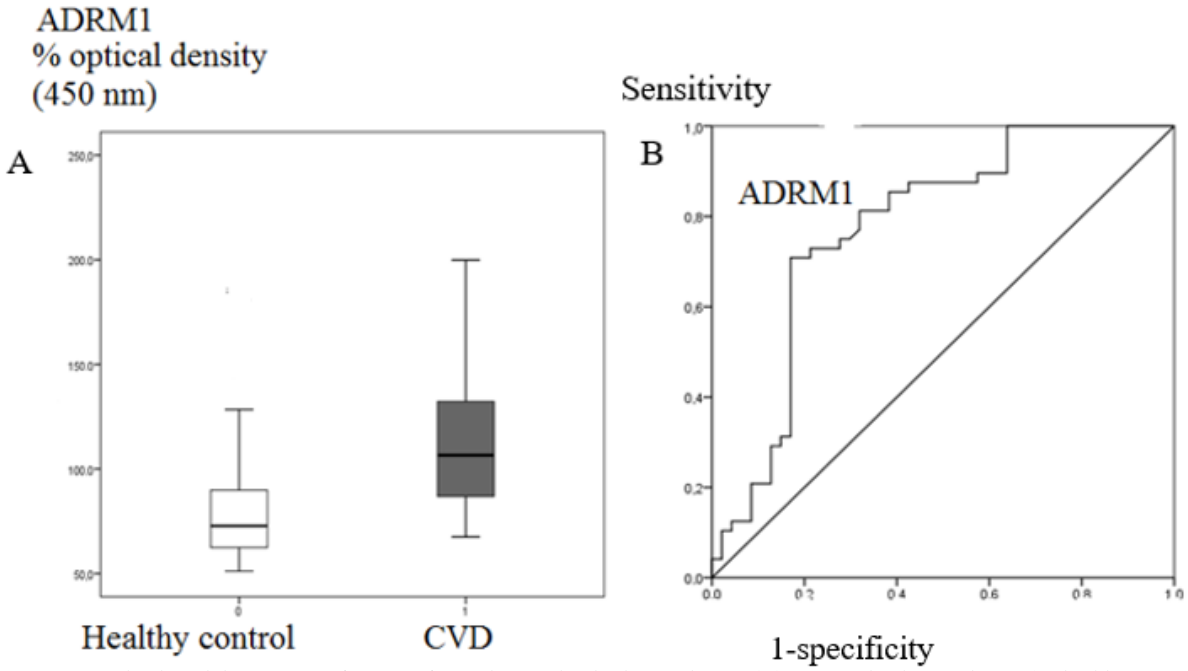

Figure 2. ADRM1 levels and diagnostic performance for cardiovascular death in Cohort 1. (A) ADRM1 levels in Cohort 1 (48 healthy controls versus 48 CVD cases). Mean % OD_{450} was 85.4 ± 35.6 in controls and 114.7 ± 36.4 in CVD cases. Welch's *t*-test *p*-value = 0.0001; Brown–Forsythe test *p*-value = 0.0001. (B) ROC curve for ADRM1 as a continuous predictor of CVD (binary outcome) in Cohort 1. AUC = 0.776; 95% CI: 0.679–0.874; $p = 0.0001$.
Abbreviations: ADRM1: Adhesion regulating molecule 1; AUC: Area under the curve; CI: Confidence interval; CVD: Cardiovascular death; ROC: Receiver operating characteristic.

was the only protein with higher levels in the CVD profile and has not been previously reported in studies on cardiovascular mortality proteomics. However, ADRM1 has been used in studies of cancer and neurodegeneration to demonstrate that ADRM1 activates pathological protein degradation.[56-58] The validation data obtained using ELISA in the present study on an expanded selection of subjects indicated that high ADRM1 was an independent, relatively specific predictor of CVD linked to a 3-fold elevated risk of CVD events during 6.5-year follow-up. High ADRM1 was considered a specific predictor of CVD because we were unable to detect ADRM1 associations with the presence of atherosclerosis or with AMI.

The mechanistic relationship between ADRM1 and

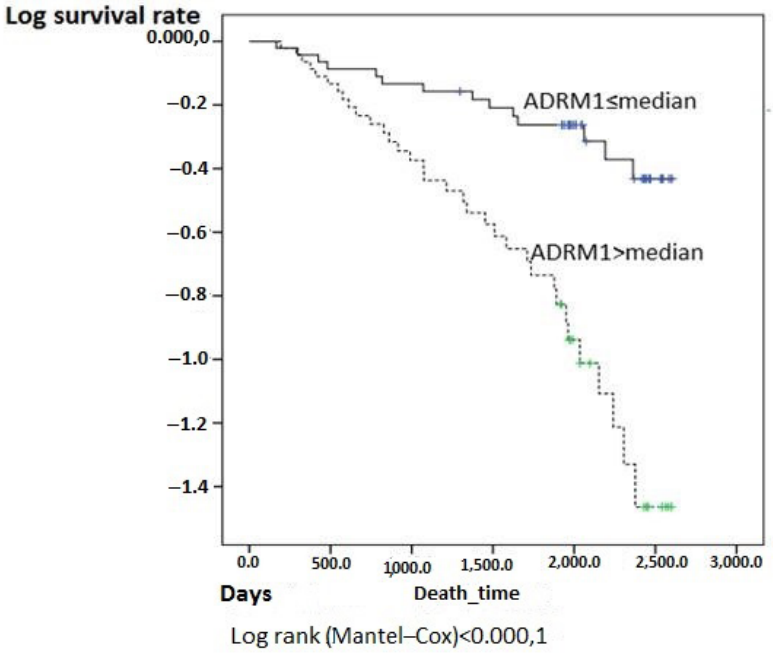

Figure 3. Kaplan–Meier survival analysis comparing participants with high versus low serum ADRM1 levels (median cut-off) in patients with CVD during follow-up and matched healthy subjects from Cohort 1.
Abbreviations: ADRM1: Adhesion regulating molecule 1; CVD: Cardiovascular death.

atherosclerosis is poorly understood. The literature suggests that ADRM1 may promote atherosclerosis progression by facilitating endothelial–mesenchymal transition and endothelial cell apoptosis as a consequence of low-density lipoprotein oxidation. Oxidative stress is the key point that activates these events.[58]

The inflammatory signaling pathway is known to be of utmost importance for atherosclerosis. The results of the present study indicated that a severe atherosclerosis profile was associated with active vascular inflammation evidenced by upregulation of inflammatory cytokines (IL-1α,[34] IL-6, and TNFα[35,41]), the NF-κB pathway components, growth factors (FGF-1, FGF-2,[38] G-CSF,[21] and GM-CSF), and matrix remodeling proteins (collagen II and MMP-16). This pattern is consistent with the results of large-scale proteomic studies, including the KORA F4 study, which identified galectin-4 as a key protein associated with coronary heart disease (CHD), and implicated inflammatory pathways in disease development.[5] Similarly, the LIPID trial demonstrated that plasma biomarkers, including C-reactive protein, are independently associated with cardiovascular death risk for up to 15 years in patients with chronic CHD.[4] In contrast, the CVD profile assessed in the present study showed a downregulation of immune mediators (neutrophil elastase, CD23, and G-CSF), which is consistent with a large-scale proteomic study of 8,343 individuals from the Generation Scotland cohort. The latter study identified 48 serum proteins significantly associated with CVD and all-cause mortality, with immune and oxidative stress responses being the most prominent features.[59]

Notably, both pro-apoptotic (caspase 1, caspase 8, and p73[43]) and anti-apoptotic (SODD and Bcl-6) factors were elevated in the severe atherosclerosis profile in the present study, and these proteins are known to be associated with a tightly regulated balance between cell death and survival. This pattern may represent a compensated inflammatory state in which at least some protective mechanisms remain active in atherosclerosis. Several apoptotic proteins were downregulated in the CVD profile (TRADD[24] and caspase 6[27,41]); however, lower levels of FLIP, an anti-apoptotic caspase-8 inhibitor, are known to be associated with pro-apoptotic signaling. These observations contrast with the severe atherosclerosis profile observed in the present study, where anti-apoptotic signaling was presented by a different factor, SODD. We propose that lower levels of FLIP suggest a particularly critical switch to activate pro-apoptotic signal transduction.[25]

Structural integrity pathways are of particular importance in cardiovascular diseases. Lower levels of structural proteins (vinculin,[15] laminin B1, and dysferlin[18]) in the CVD profile may result in compromised cellular and

extracellular integrity. This finding is supported by data from the ARIC study, which demonstrated that extracellular structural processes were associated with sudden cardiac death and identified Sushi, von Willebrand factor type A, EGF, and pentraxin domain-containing protein 1, an extracellular matrix and cell adhesion protein, as a potential predictor of sudden cardiac death.[5] A proteome-wide Mendelian randomization study identified 28 plasma proteins associated with atherosclerosis.[60] The latter study was focused on atherosclerosis development rather than on terminal CVD events, although the patterns of protein upregulation in active disease and downregulated levels in the terminal stages were consistent with observations of the present study that detected lowered levels of dysferlin and G-CSF.[59]

Decreased levels of calmodulin and S100 in the CVD profile in the present study point out that calcium signaling may represent a novel pathway in predictive CVD mortality proteomics.

Chronic kidney disease (CKD)-related systemic alterations may partially mimic or amplify features interpreted here as terminal cardiovascular collapse. Although participants with CKD were excluded, residual confounding related to renal function cannot be completely ruled out. Circulating protein and metabolite concentrations are influenced not only by manifested CKD but also by physiological variation in glomerular filtration, tubular secretion, tubular reabsorption, and renal metabolism.[61] Consequently, subtle differences in kidney function among individuals without CKD may still affect serum proteomic profiles and contribute to the observed associations. Therefore, the proposed mechanistic interpretation should be considered within this broader biological context.

Thus, our findings revealed the central role of inflammatory and structural proteins, supporting the biological validity of our observations.

The main limitation of the present study is its cross-sectional and cross-cohort design, which limits our ability to establish causality. Thus, all conclusions and data may only point to associations.

Another major limitation is the relatively small sample size (n = 9–10 per group) for proteomic profiling. This limitation is mainly due to technical problems and the high costs of antibody-based microarrays used for profiling. Thus, the results of multidimensional analysis should be interpreted with caution and may reflect false positives, false negatives, and instability of associations in expanded groups. However, we think that the statistical power estimates for detection of single protein differences should be valid. Notably, in addition to the Holm–Bonferroni correction, we used FDR corrections, volcano plots, principal component analysis, hierarchical clustering, and other methods of evaluation that confirmed the data obtained using the standard Holm–Bonferroni correction to exclude bias, which we thus presume to be minimal. Additionally, we have confirmed ADRM1 data using an independent ELISA, and have previously confirmed the validity of differences detected by antibody microarray profiling in our previous publications.[12] Finally, we have not assessed the functional activities of the pathways analyzed in the present study and rely entirely on up- or down-regulation of pathway components and other studies described in the literature to estimate the impact of our data.

Another potentially important limitation is the use of pharmacotherapy by the participants. Unfortunately, this information was not available with sufficient reliability for Cohorts 1 and 2. However, the information was available for Cohort 3, where the data indicated a lack of correlation between ADRM1 levels and use of statins and other major cardiovascular drugs (data not shown).

Thus, the limited size of discovery proteomic groups may be acceptable mainly for hypothesis generation.

5. Conclusion

In the present study, patients who died from cardiovascular events during 6.5-year follow-up had lower levels of proteins involved in apoptosis, inflammation, cell adhesion, proliferation, and calcium signaling. In contrast, patients with CVD had significantly higher levels of ADRM1, suggesting that this protein may be an independent specific predictor of CVD during follow-up. Severe atherosclerosis and CVD during follow-up were characterized by substantially different proteomic profiles. Severe atherosclerosis was associated with active compensated inflammation with balanced apoptotic signaling and active repair mechanisms. In contrast, CVD was associated with loss of structural integrity, immune system and calcium signaling dysregulation, pro-apoptotic signaling, and enhanced proteasomal degradation. These findings indicate that CVD during follow-up may involve failure of multiple protective systems. Understanding this transition may open new therapeutic approaches to prevent cardiovascular disease progression.

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Conflict of interest

The authors declare they have no competing interests.

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Ethics approval and consent to participate

The study was approved by the NRCPM Independent Ethics Committee (approval no. 09–05/19 and approval no. 07-05/12) and conducted in accordance with the Declaration of Helsinki and WHO guidelines. Written informed consent was obtained from all participants.

Consent for participation

Verbal consent was obtained from each subject to publish their data.

Availability of data

Data utilized in this study have been uploaded onto Mendeley, as follows:

- Gumanova N. Proteomics-Gumanova. Mendeley Data, V1; 2026. doi: 10.17632/mpfcxbp839.1
- Gumanova N. Base-Gumanova. Mendeley Data, V1; 2026). doi: 10.17632/x48yww3m4j.1
- Gumanova N. Novel relationships between the ICAD, CD53, GRM1, and GSTmu protein levels and atherosclerosis and cardiovascular outcomes. Mendeley Data, V1; 2025. doi: 10.17632/vktn6sfbr2.1

Further disclosure

Portions of this manuscript were edited using DeepSeek AI tools solely for language refinement. The authors have thoroughly examined and confirmed all artificial intelligence-generated outputs and assume complete responsibility for the scientific validity of the manuscript.

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