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Serum Biomarkers that Stimulate the Mitogen-Activated Protein Kinase Cascade in Relation to Recurrent Coronary Events Following an Acute Coronary Syndrome

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Abstract

Background: The intracellular mitogen-activated protein kinase (MAPK) cascade regulates intracellular processes that modulate cardiovascular disease progression. We explored the time-course of serum biomarkers that stimulate the MAPK-cascade in post-acute coronary syndrome (ACS) patients, prior to a recurrent coronary event.

Methods: BIOMArCS is a high-frequent repeated blood sampling study in post-ACS patients. We performed a nested case-control study selecting the 45 patients who experienced a recurrent event (cases) and 2 matched event-free controls per case during 1-year follow-up. Olink Proteomics 'immunoassay was used to measure 25 serum biomarkers. Results are expressed in the arbitrary Normalized Protein eXpression (NPX) unit on the 2log-scale. Linear mixed-effects models were applied to examine time-courses and differences between cases and controls.

Results: Mean age was 66 ± 12 years and 80% were men, with no differences between cases and controls. Early cases had significantly higher levels of ANG-1 (difference 0.95 NPX (95%CI 0.36-1.54), PAR-1 (difference 0.50 NPX (95%CI 0.22-0.77) and BMP-6 (difference 0.55 NPX (95%CI 0.21-0.90) than controls. No differences in biomarker levels were observed between late cases and matching controls. In particular, in cases, no increase was observed prior to the moment of the recurrent event.

Conclusion: Patients with an early recurrent coronary event after an index-ACS had higher levels of ANG-1, PAR-1 and BMP-6 than patients who remained event-free.

Keywords: Acute coronary syndrome; Mitogen-activated protein kinase; Proteomics; Proteins; Repeated measurements; Temporal pattern

Abbreviations: ACS: Acute Coronary Syndrome; ANG-1: Angiopoietin-1; BIOMArCS: BIOMarker study to identify the Acute risk of a Coronary Syndrome; BMP-6: Bone Morphogenetic Protein 6; CI: Confidence Interval; CVD: Cardiovascular Disease; IQR: Interquartile Range; MAPK: Mitogen-Activated Protein Kinase; MI: Myocardial Infarction; NPX: Normalized Protein eXpression; PAR-1: Proteinase-Activated Receptor 1; PEA: Proximity Extension Assay; UA: Unstable Angina

Introduction

Although outcome for patients with cardiovascular disease (CVD) has improved over the last decades, hospitalization rates are still increasing [1-3]. This increase can partly be explained by the increasing population of patients who survived an acute coronary syndrome (ACS) and who are at risk of experiencing a recurrent coronary event [2]. As CVD is dynamic and shows considerable inter-patient variation, to improve secondary prevention, insight in the course of CVD in individual subjects is required. Since biomarker profiles may serve as a proxy for CVD status and development, the

exploration of (established and) evolving markers covering relevant pathophysiological processes is warranted.

The mitogen-activated protein kinase (MAPK) cascade, is an intracellular cascade of proteins that enables extracellular stimuli – blood biomarkers - to modulate several intracellular processes i.e., cell growth, differentiation, proliferation, and apoptosis [4]. Basic research has shown that the MAPK-cascade plays a pivotal role in cellular processes that advance CVD progression [5]. First, the MAPK-cascade promotes atherosclerotic lesion formation, i.e., by inducing inflammation and cell apoptosis. Secondly, it may activate pathological cardiac remodeling after myocardial infarction by constraining myocyte mitosis and promoting fibrosis. Thirdly, the cascade might be directly involved in the development of in-stent restenosis [5].

In view of the pivotal role of the MAPK-cascade in the development and progression of CVD, blood biomarkers that regulate the cascade may be useful for the identification of patients with CVD who are at higher risk of developing a (recurrent) coronary event. However, translational research relating the MAPK-cascade to clinical CVD progression is scarce [5].

We aimed to explore the course of protein blood biomarkers that stimulate the intracellular MAPK-cascade in post-ACS patients prior to the development of a recurrent coronary event during one year of follow-up.

Materials and Methods

Study population

We performed a case-control study that is embedded in The 'BIOMarker study to identify the Acute risk of a Coronary Syndrome' (BIOMArCS) [6]. BIOMArCS is a multicenter observational study with a unique high-frequency sampling design, to study the course of blood biomarkers in patients following an ACS in anticipation of a recurrent event. The design of BIOMArCS has been described in detail elsewhere [6]. In brief, BIOMArCS enrolled 844 patients with ACS, aged ≥ 40 years and who had at least 1 pre-specified cardiovascular risk factor. After enrolment, venipuncture was performed at admission, discharge, and subsequently every two weeks during the first half-year and every month during the second half-year. A median number of 17 repeated blood samples per patient were obtained.

BIOMArCS was approved by the Institutional Review Boards of all enrolling hospitals, and all participating patients provided written informed consent. BIOMArCS is registered in The Netherlands Trial Register NTR1698 and NTR1106.

Case-control design

The current analysis is based on a case-control approach. A total of 45 patients (cases) in BIOMArCS reached the composite study endpoint of cardiac death, non-fatal myocardial infarction (MI), or unstable angina (UA) requiring urgent coronary revascularization during one year of follow-up after the index-ACS. These cases were with two controls that are selected from BIOMArCS event-free patients. Cases and controls were matched on age, sex and admitted hospital. For reasons of efficiency, for each case, the blood sample at hospital admission and the last and second last samples prior to the recurrent coronary event have been analyzed. In controls, we selected the blood sample at hospital admission and the blood sample that corresponds in time with the recurrent event of the matched case.

As a pragmatic choice, separate analyses were performed for cases (and their matching controls) that experienced their event in the first 30 days after the index-ACS, and for cases (and their matching controls) that experienced their event thereafter. Hence, we were able to differentiate between the behavior of biomarkers during the acute and post-stabilization phase after the index-ACS.

Biomarker measurements

Targeted protein biomarker measurements were performed by the Proximity Extension Assay (PEA) Technique using Olink Proteomics' CVD II panel (Olink Proteomics AB, Uppsala, Sweden). Details concerning PEA and the CVD II panel are described on the website of Olink Proteomics (www.olink.com). In brief, the PEA technique consists of a pair of oligonucleotide-labelled antibody probes that pair-wise bind to a targeted protein biomarker in a blood sample. This binding induces amplification of the protein biomarker by real-time PCR (Fluidigm® BioMark™ HD System).

The PEA technique enables simultaneous analysis of all protein biomarkers of the CVD II panel in one blood sample. Olink proteomics' CVD II panel provides measurements of 25 protein blood biomarkers that are related to the intracellular MAPK-cascade (overview of proteins in Table 1). Every measured protein blood biomarker is expressed in an arbitrary unit on the log2-scale called Normalized Protein eXpression (NPX). Accordingly, an increase or decrease of one NPX corresponds with a doubling or halving of a biomarker serum level. NPX values cannot be compared across different proteins. For each protein biomarker, general calibrator curves to calculate approximate concentrations are available on the website of Olink Proteomics.

Statistical analysis

Continuous variables are presented as medians with interquartile range (IQR) and categorical variables as numbers with percentages. Differences between cases and controls were compared with Mann-Whitney U and Pearson Chi-square tests, respectively.

As indicated, all biomarkers were analyzed on a log2-transformed scale. We fitted a linear mixed-effects model for every biomarker to describe patient-specific longitudinal biomarker trajectories as a function of time. Likelihood ratio tests and F-tests were used for hypothesis testing, whereas residuals were used to examine the model assumptions.

We considered 25 biomarkers. To adjust for inflation of the type I error with multiple testing, statistical significance was stated at p=0.003 (two-tailed test), based on the matrix spectral decomposition method [7]. R statistical software (version 3.4.0) was used for analyses, in particular the package nlme (https://cran.r-project.org/web/packages/nlme/index.html).

Results

Baseline characteristics

Mean age of all patients was 66.0 ± 11.9 years and 80.2% were men. Cases and controls did not show any significant differences in presentation, initial treatment, cardiovascular risk factors and medication at first blood sample, indicating successful matching (Table 2).

Biomarker trajectories in the first 30 days after the index ACS

Fifteen cases reached the study endpoint within 30 days after the index ACS. They had higher serum levels of ANG-1 (difference of 0.95 NPX, 95% confidence interval [CI] 0.36-1.54), PAR-1 (difference of 0.50 NPX, 95%CI 0.22-0.77) and BMP-6 (difference of 0.55 NPX, 95%CI 0.21-0.90) than the matched controls (Table 3, Figure 1 left-hand panel).

Abbreviation	Full name	Synonyms	Molecular function
NEMO	NF-kappa-B essential modulator	IKBKG, FIP3	Binding protein
HB-EGF	Proheparin-binding EGF-like growth factor	DTR, DTS, HEGFL	Growth factor/receptor
SCF	Stem cell factor	KITLG, MGF	Cytokine/growth factor
PDGF subunit B	Platelet-derived growth factor subunit B	PDGFB, PDGF2, SIS	Developmental protein/growth factor
GDF-2	Growth/differentiation factor 2	BMP9	Cytokine/growth factor
ANG-1	Angiopoietin-1	ANGPT1	Developmental protein
CCL3	C-C motif chemokine 3	MIP1A, SCYA3, G0S19-1	Chemokine
TIE2	Angiopoietin-1 receptor	TEK, VMCM, VMCM1	Receptor
PAR-1	Proteinase-activated receptor 1	F2R, CF2R, TR	Receptor
LEP	Leptin	OB, OBS	Hormone/growth factor
REN	Renin		Hydrolase
TNFRSF11A	Tumor necrosis factor receptor superfamily member 11A	RANK, ODFR, CD265, NFKB activator	Receptor
THPO	Thrombopoietin/megakaryocyte colony-stimulating factor	MGDF	Cytokine/hormone
FGF-21	Fibroblast growth factor 21		Growth factor
GAL-9	Galectin-9	LGALS9	Binding protein
SRC	Proto-oncogene tyrosine-protein kinase SRC	SRC1, C-SRC	Kinase
GH	Growth hormone	GH1, somatotropin	Hormone
XCL1	X-C motif chemokine ligand 1	Lymphotactin, LTN, SCYC1, ATAC, SCM-1	Chemokine
FGF-23	Fibroblast growth factor 23	HYPF	Growth factor
CCL17	C-C motif chemokine 17	SCYA17, TARC	Chemokine
IL-18	Interleukin-18	IGIF, IL1F4	Cytokine
BMP-6	Bone morphogenetic protein 6	VGR1, VGR	Cytokine/developmental protein/growth factor
IL-6	Interleukin-6	IFNB2, BSF2, CDF, HGF	Cytokine/growth factor
AMBP	alpha-1-microglobulin/bikunin precursor	HCP, ITIL, ITI, Bikunin, EDC1, Trypstatin	Protease inhibitor
CD40-L	CD40 ligand	CD40LG, TNFSF5, TRAP, HIGM1, CD154	Cytokine

Table 1: Overview of the assessed protein biomarkers.

Biomarker trajectories after 30 days

Thirty cases had the study endpoint in >30 days after the index ACS. Interestingly, in these late cases, the biomarker levels during the first 30 days after the index ACS tended to be lower than in the early cases (Table 4). In the post-30 day time window, cases and matched controls appeared to have similar levels of biomarkers (Table 5). Importantly, we found no steady or sudden increase in biomarkers in the days or weeks prior to the recurrent event.

Discussion

Serum levels of ANG-1, PAR-1 and BMP-6 were significantly higher in patients who developed a recurrent coronary event within the first

30 days following an ACS than in their matching controls. In the time period >30 days after the index-ACS until 1 year follow-up, patients with and without a recurrent coronary event had similar patterns of MAPK stimulating biomarkers.

	Cases	Controls	p-value
	n=44	n=87	
Presentation and initial treatment			
Men	35 (79.5)	70 (80.5)	0.9
Age - yr	67.5 (57.3-77.5)	66.7 (57.4-75.5)	0.83
Admission diagnosis	-	-	0.38
STEMI	16 (36.4)	42 (48.3)	-
NSTEMI	22 (50.0)	33 (37.9)	-
UAP	6 (13.6)	12 (13.8)	-
CAG performed	39 (88.6)	82 (94.3)	0.25
PCI performed	33 (86.8)	67 (81.7)	0.48
CKmax - U/L	418 (195-1142)	513 (169-1332)	0.94
Cardiovascular risk factors			
Smoking	-	-	0.81
Current	17 (38.6)	35 (40.2)	-
Former	12 (27.3)	27 (31.0)	-
Never	15 (34.1)	25 (28.7)	-
Diabetes mellitus	16 (36.4)	32 (36.8)	0.96
Hypertension	21 (47.7)	44 (50.6)	0.76
Hypercholesterolemia	19 (43.2)	46 (52.9)	0.3
Creatinine - µmol/L	88 (73-93)	81 (67-97)	0.15
Cardiovascular history			
Peripheral arterial disease	10 (22.7)	7 (8.0)	0.018
Myocardial infarction	14 (31.8)	33 (37.9)	0.49
PCI	14 (31.8)	29 (33.3)	0.86
CABG	10 (22.7)	17 (19.5)	0.67
Stroke	9 (20.5)	5 (5.7)	0.01
Valvular heart disease	4 (9.1)	3 (3.4)	0.18
Heart failure	4(9.1)	1 (1.1)	0.025
Medication at first blood sample moment >7 days after the index ACS *			
Aspirin	35 (92.1)	76 (92.7)	0.91
P2Y12 inhibitor	36 (94.7)	74 (90.2)	0.41
Vitamin K antagonist	7 (18.4)	8 (9.8)	0.18
Statin	35 (92.1)	79 (96.3)	0.32
Beta-blocker	36 (94.7)	69 (84.1)	0.1
ACE inhibitor or ARB	34 (89.5)	65 (79.3)	0.17

Continuous variables are presented as median (25th-75th percentile). Categorical variables are presented as number (percentage). *The first blood sample >7 days was taken at a median (25th-75th percentile) of 24 (16-34) days after the index ACS.

ACE: Angiotensin Converting Enzyme; ARB: Angiotensin II Receptor Blocker; CABG: Coronary Artery Bypass Grafting; CKmax: Maximum Creatine Kinase during the index admission; NSTEMI: Non-ST-Elevation Myocardial Infarction; PCI: Percutaneous Coronary Intervention; STEMI: ST-Elevation Myocardial Infarction; Troponin ax: Maximum Troponin value during the index admission; UAP: Unstable Angina Pectoris; yr: years.

Table 2: Baseline clinical characteristics.

Our study results suggest that during the first 30 days post-ACS, the initial ACS induces numerous stimuli that activate the intracellular MAPK-cascade, which, in turn, may induce a pro-inflammatory and thrombogenic state, leading to a recurrent event. Potentially, other processes play a more important role in the initiation of a new coronary event following stabilization after the first 30 days post-ACS.

PAR-1 is a receptor expressed by cardiomyocytes, fibroblasts, smooth muscle cells and vascular endothelial wall cells [8]. Basic scientific research showed that PAR-1 may stimulate pathological remodeling after cardiac ischemia/reperfusion injury [8,9]. Moreover, PAR-1 is involved in hemostasis and thrombosis [8,10,11]. PAR-1 modulates thrombin signaling and is expressed on platelets, and may activate platelet secretion and aggregation. Local tissue injury of the vascular endothelial wall might induce endothelial responses via PAR-1, like recruitment of leukocytes and platelets, to manage infection or damage [11]. Accordingly, higher PAR-1 serum levels during the first 30 days following an ACS, might play a role in pathological remodeling after an ACS, and may lead to the development of (platelet-dependent) arterial thrombosis, and thus the recurrence of a coronary event.

Biomarker (NPX)	Coefficient	95%CI	p-value
NEMO	1.16	(0.36-1.95)	0.005
HB-EGF	0.61	(0.11-1.12)	0.018
SCF	-0.13	(-0.59-0.32)	0.55
PDGF subunit B	0.92	(0.31-1.54)	0.004
GDF-2	-0.063	(-0.35-0.23)	0.66
ANG-1	0.95	(0.36-1.54)	0.002
CCL3	0.29	(-0.12-0.71)	0.16
TIE2	0.12	(-0.048-0.29)	0.16
PAR-1	0.5	(0.22-0.77)	<0.001
LEP	0.35	(-0.31-1.00)	0.29
REN	0.45	(-0.18-1.08)	0.16
TNFRSF11A	0.41	(0.003-0.82)	0.048
THPO	0.22	(-0.018-0.45)	0.069
FGF-21	0.55	(-0.39-1.49)	0.24
GAL-9	0.16	(-0.083-0.41)	0.19

SRC	0.29	(-0.18-0.76)	0.22
GH	0.71	(-0.61-2.02)	0.29
XCL1	0.34	(0.018-0.66)	0.039
FGF-23	0.9	(0.16-1.63)	0.018
CCL17	0.77	(0.089-1.46)	0.028
IL-18	0.19	(-0.15-0.52)	0.27
BMP-6	0.55	(0.21-0.90)	0.002
IL-6	0.95	(0.074-1.82)	0.034
AMBP	0.11	(-0.033-0.25)	0.13
CD40-L	1.32	(0.41-2.22)	0.006
For every biomarker, the difference in biomarker serum level between cases and controls is expressed in a relative arbitrary unit on the log 2 scale. Thus, an increase or decrease of one NPX corresponds with a doubling or a halving of the protein biomarker serum level.			
ACS: Acute Coronary Syndrome, CI: Confidence Interval, NPX: Normalized Protein eXpression			

Table 3: Difference in biomarker serum level between cases and controls ≤ 30 days.

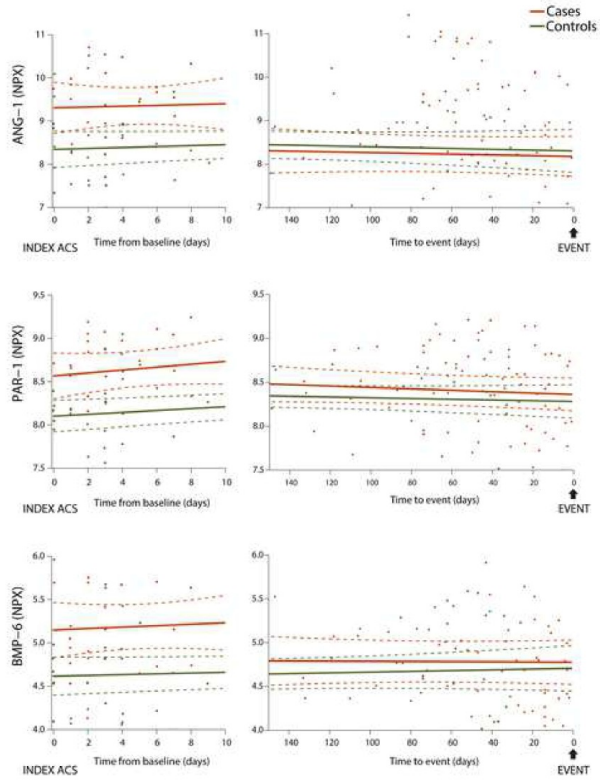

Figure 1: Time-course of PAR-1, BMP-6 and ANG-1.

Biomarker (NPX)	Early cases*	Late cases*	p-value
NEMO	6.61 ± 1.08	5.80 ± 1.25	0.062
HB-EGF	5.62 ± 0.89	5.03 ± 0.73	0.049
SCF	8.31 ± 0.87	8.08 ± 0.60	0.4
PDGF subunit B	10.47 ± 0.84	9.49 ± 1.16	0.012
GDF-2	3.16 ± 0.55	2.94 ± 0.37	0.19
ANG-1	9.31 ± 0.91	8.38 ± 1.21	0.022
CCL3	4.04 ± 0.59	3.59 ± 0.36	0.013
TIE2	7.02 ± 0.19	6.86 ± 0.39	0.16
PAR-1	8.66 ± 0.35	8.26 ± 0.48	0.012
LEP	5.26 ± 0.93	4.94 ± 0.51	0.23
REN	8.36 ± 1.34	8.31 ± 1.06	0.91
TNFRSF11A	5.55 ± 0.68	5.15 ± 0.61	0.084
THPO	2.99 ± 0.33	2.87 ± 0.80	0.6
FGF-21	7.01 ± 1.51	6.72 ± 1.12	0.54
Gal-9	7.93 ± 0.39	7.79 ± 0.39	0.34
SRC	7.20 ± 0.37	7.06 ± 0.55	0.4
GH	8.02 ± 2.14	7.27 ± 1.96	0.31
XCL1	4.77 ± 0.52	4.79 ± 0.49	0.92
FGF-23	4.02 ± 1.77	2.84 ± 0.63	0.025
CCL17	8.74 ± 1.14	8.57 ± 1.26	0.7
IL-18	8.59 ± 0.58	8.36 ± 0.46	0.21
BMP-6	5.23 ± 0.60	4.80 ± 0.77	0.091
IL-6	6.00 ± 1.86	4.89 ± 1.37	0.062
AMBP	6.00 ± 0.22	5.90 ± 0.23	0.26
CD40-L	7.28 ± 1.41	6.28 ± 1.44	0.058
Blood samples ≤ 30 days after the index ACS were available for 15 ≤ 30 days cases and 17 >30 days cases.			
*Patient-level mean value ± standard deviation			
NPX: Normalized Protein eXpression			

Table 4: Biomarker serum levels in the first 30 days for cases only

BMP-6 is part of the transforming growth factor β family of cytokines. BMP-6 is involved in activation of osteogenic markers in mesenchymal stem cells, and may modulate ectopic cartilage and bone matrix formation [12]. Bone matrix formation is one of the key processes responsible for vascular calcification [13]. Since BMPs are overexpressed in (vulnerable) atherosclerotic lesions, it is suggested that BMPs modulate vascular calcification [14]. Furthermore, it is observed that BMPs contribute to vascular inflammation [14-16]. Lastly, previous research has indicated that oxidative stress may induce BMP-6 expression and thereby vascular inflammation and calcification [12]. Thus, it could be hypothesized that post-ACS oxidative stress may

induce higher BMP-6 serum levels, which – in turn - might induce vascular inflammation and a recurrent coronary event.

	Median maximum value ≤ 7 days[a]		Patient-level mean value ≤ 30 days[b]		Patient-level mean value >30 days[b]	
Biomarker (NPX)	**Cases**	**Controls**	**Cases**	**Controls**	**Cases**	**Controls**
NEMO	6.50 (5.51-7.29)	5.75 (4.70-6.63)	6.18 ± 1.22	5.67 ± 1.33	5.44 ± 1.58	5.66 ± 1.50
HB-EGF	5.43 (4.73-5.82)	5.06 (4.48-5.60)	5.31 ± 0.85	5.15 ± 0.88	5.19 ± 0.89	5.32 ± 0.95
SCF	8.18 (7.45-8.88)	8.35 (7.99-8.77)	8.19 ± 0.74	8.40 ± 0.54	8.45 ± 0.55	8.53 ± 0.45
PDGF subunit B	10.66 (9.37-10.87)	10.39 (8.95-10.69)	9.95 ± 1.12	9.72 ± 1.20	9.37 ± 1.45	9.72 ± 1.34
GDF-2	3.33 (3.74-3.59)	3.26 (2.97-3.60)	3.04 ± 0.47	3.28 ± 0.41	3.43 ± 0.48	3.49 ± 0.42
ANG-1	9.51 (7.86-9.78)	8.75 (7.72-9.54)	8.81 ± 1.17	8.52 ± 1.16	8.20 ± 1.25	8.51 ± 1.19
CCL3	3.97 (3.48-4.22)	3.55 (3.21-4.12)	3.80 ± 0.52	3.70 ± 0.68	3.59 ± 0.55	3.62 ± 0.52
TIE2	7.01 (6.89-7.19)	6.88 (6.71-7.17)	6.93 ± 0.32	6.93 ± 0.31	7.01 ± 0.27	6.96 ± 0.32
PAR-1	8.69 (8.27-8.88)	8.36 (7.88-8.59)	8.45 ± 0.47	8.25 ± 0.51	8.40 ± 0.48	8.37 ± 0.51
LEP	5.09 (4.58-5.73)	5.00 (3.96-5.79)	5.09 ± 0.74	4.90 ± 1.22	5.06 ± 0.86	4.96 ± 1.09
REN	8.74 (7.21-9.88)	7.88 (7.05-8.75)	8.34 ± 1.18	8.08 ± 1.06	8.47 ± 1.01	8.24 ± 1.00
TNFRSF11A	5.44 (5.04-6.01)	5.10 (4.60-5.51)	5.34 ± 0.67	5.10 ± 0.69	5.22 ± 0.66	5.09 ± 0.59
THPO	3.01 (2.75-3.42)	2.66 (2.35-2.97)	2.93 ± 0.62	2.72 ± 0.48	2.70 ± 0.65	2.67 ± 0.44
FGF-21	7.33 (6.71-8.36)	6.36 (5.26-7.59)	6.86 ± 1.31	6.60 ± 1.51	7.14 ± 1.21	6.48 ± 1.70
GAL-9	7.94 (7.64-8.10)	7.78 (7.42-8.05)	7.86 ± 0.39	7.79 ± 0.45	7.86 ± 0.37	7.84 ± 0.40
SRC	7.31 (7.21-7.44)	7.34 (7.00-7.61)	7.13 ± 0.47	7.02 ± 0.83	6.77 ± 0.99	6.92 ± 0.93
GH	9.07 (6.46-9.76)	7.15 (5.93-8.92)	7.62 ± 2.05	7.52 ± 2.01	7.32 ± 1.92	7.72 ± 2.05
XCL1	4.72 (4.50-5.21)	4.49 (4.19-4.87)	4.78 ± 0.50	4.53 ± 0.55	4.73 ± 0.57	4.60 ± 0.55
FGF-23	3.32 (2.78-3.89)	2.80 (2.55-3.24)	4.02 ± 1.77	2.93 ± 0.71	3.18 ± 0.81	3.03 ± 0.70
CCL17	8.53 (8.21-9.64)	8.12 (7.57-8.97)	8.74 ± 1.14	8.32 ± 1.39	8.47 ± 1.30	8.46 ± 1.29
IL-18	8.67 (8.07-9.00)	8.50 (7.97-8.76)	8.59 ± 0.58	8.44 ± 0.59	8.37 ± 0.41	8.50 ± 0.56
BMP-6	4.96 (4.65-5.69)	4.61 (4.14-5.11)	5.23 ± 0.60	4.68 ± 0.59	4.77 ± 0.69	4.67 ± 0.62
IL-6	6.56 (4.42-7.18)	5.08 (4.24-5.75)	6.00 ± 1.86	4.63 ± 1.42	3.99 ± 1.04	3.69 ± 0.91
AMBP	5.99 (5.82-6.18)	5.94 (5.68-6.07)	5.99 ± 0.22	5.87 ± 0.24	5.97 ± 0.22	5.89 ± 0.22
CD40-L	7.01 (6.17-8.31)	6.31 (5.40-7.29)	7.28 ± 1.41	6.75 ± 1.49	6.11 ± 1.62	6.37 ± 1.66
Blood samples ≤ 7, ≤ 30 and >30 days after the index ACS were available for 23, 32, 28 cases and for 44, 67, 70 controls.						
a. Median (25th-75th percentile) value of the patient-level maximum. b. Mean ± standard deviation value of the patient-level mean.						
NPX: Normalized Protein eXpression.						

Table 5: Biomarker serum levels in relation to time post index-ACS.

ANG-1 is a widely expressed biomarker and is involved in multiple cellular processes that occur following an ACS [17-19]. ANG-1 modulates endothelial cell survival, proliferation, migration and reorganization. Furthermore, it promotes angiogenesis and vascular quiescence. However, in the absence of vascular endothelial growth factor (VEGF) exposure, ANG-1 may promote vessel regression [18]. To the contrast of our study, previous research indicated that ANG-1 positively modulates cardiovascular disease, and promotes cardiomyocyte survival and reduces infarct size [20-22]. Furthermore, one study showed that a lower serum level of ANG-1 on admission

date, significantly predicted the development of one-year major cardiovascular events in post-ACS patients [21].

Despite the complexity of the process, it is of interest to study blood biomarkers that stimulate the intracellular MAPK-cascade, since they may serve as novel biomarkers for aggravation of CVD. Because of the exploratory character of this study, our study results are limited to the examination of divergent biomarker patterns and thus, primarily, are valuable for exploration and identification of (novel) protein blood biomarkers. Further research is needed to establish whether the studied protein blood biomarkers may actually be used to identify post-ACS patients who are at higher risk of developing a recurrent coronary event.

Limitations

Since we chose to use Olinks'Proteomics PEA high throughput analysis to efficiently analyze our samples for potential discovery of novel protein blood biomarkers, our study results lack generalizability because of the use of arbitrary units. In addition, although Olinks'Proteomics PEA is an assay that gives highly reproducible results [23]. PEA technique still needs improvements to assure complete reproducibility. Lastly, because of the small number of events in our study, differences between cases and controls may have been masked.

Conclusion

In conclusion, the serum levels of ANG-1, PAR-1 and BMP-6, all biomarkers that stimulate the MAPK-cascade, were significantly elevated in patients with ACS who developed an early recurrent coronary event. These signaling proteins warrant further study on their potential use as novel biomarkers to identify high risk post-ACS patients.

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