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ORIGINAL ARTICLE

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The temporal pattern of immune and inflammatory proteins prior to a recurrent coronary event in post-acute coronary syndrome patients

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ABSTRACT

Purpose: We assessed the temporal pattern of 29 immune and inflammatory proteins in post-acute coronary syndrome (ACS) patients, prior to the development of recurrent ACS.

Methods: High-frequency blood sampling was performed in 844 patients admitted for ACS during one-year follow-up. We conducted a case-control study on the 45 patients who experienced reACS (cases) and two matched event-free patients (controls) per case. Olink Proteomics' immunoassay was used to obtain serum levels of the 29 proteins, expressed in an arbitrary unit on the log2-scale (Normalized Protein eXpression, NPX). Linear mixed-effects models were applied to examine the temporal pattern of the proteins, and to illustrate differences between cases and controls.

Results: Mean age was 66 ± 12 years and 80% were men. Cases and controls had similar baseline clinical characteristics. During the first 30 days, and after multiple testing correction, cases had significantly higher serum levels of CXCL1 (difference of 1.00 NPX, $p = 0.002$), CD84 (difference of 0.64 NPX, $p = 0.002$) and TNFRSF10A (difference of 0.41 NPX, $p < 0.001$) than controls. After 30 days, serum levels of all 29 proteins were similar in cases and controls. In particular, no increase was observed prior to reACS.

Conclusions: Among 29 immune and inflammatory proteins, CXCL1, CD84 and TNFRSF10A were associated with early reACS after initial ACS-admission.

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KEYWORDS
Acute coronary syndrome; biomarkers; immune and inflammatory system; proteins; proteomics; temporal pattern

Introduction

In the pathophysiology of atherosclerosis, the lipid metabolism and the immune and inflammatory systems are interconnected (Libby *et al.* 2009, 2011). It is known that both lipids and inflammatory biomarkers are affected by LDL lowering treatment, which importantly reduces the occurrence of cardiovascular events. However, despite adequately lowering LDL levels, a considerable number of patients with CVD will still develop adverse (coronary) events, especially those with a residual inflammatory risk (Libby 2005). Therefore, more insights in the role of the immune- and inflammatory systems are required.

The research field of proteomics offers a novel way to gain understanding of disease processes (Miller *et al.* 2007). As the proteome is considered the end product of the genome, and has a regulatory role in all kinds of biological processes in the human body, proteins are fundamental to determine onset and progression of diseases, including CVD. The advantage of research on proteins is the direct information it may offer at tissue level, regardless of a patient's genotype. Novel technologies are emerging to simultaneously detect expression patterns of multiple proteins. These technologies offer the opportunity to assess expression patterns of proteins belonging to several pathophysiological pathways simultaneously (Assarsson *et al.* 2014).

Studying the temporal behaviour of the proteome in patients with CVD prior to a recurrent coronary event may potentially lead to the identification of proteins related to progression of atherosclerosis. Therefore, we performed a controlled prospective study to assess the temporal pattern of a wide range of proteins involved in the immune- and inflammatory systems just prior to the recurrence of a coronary event during one year follow-up of patients admitted with an acute coronary syndrome (ACS).

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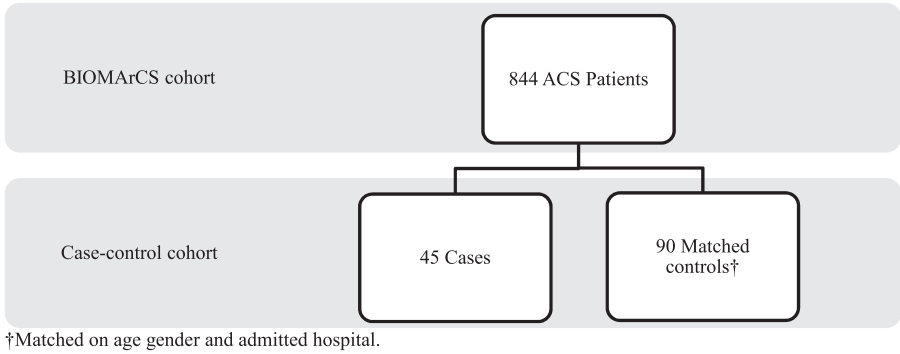

Figure 1. Patient flow diagram. BIOMArCS: BIOMarker study to identify the Acute risk of a Coronary Syndrome; ACS: acute coronary syndrome.

Clinical significance

- Studying the behaviour over time of the proteome involved in patients with ACS prior to a recurrent coronary event may lead to the identification of proteins related to progression of pathological atherogenesis.
- Our exploratory study shows that an ACS may trigger short-term upregulation of CXCL1, CD84 and TNFRSF10A which might play a role in the development of early recurrent coronary events.
- Further research, including in vitro studies, are warranted to unravel the potential mechanisms underlying our findings.

Methods

Study population

The 'BIOMarker study to identify the Acute risk of a Coronary Syndrome' (BIOMArCS) is a multicentre observational study with a design characterized by high-frequent blood sampling to assess the course over time of blood biomarkers during one-year follow-up of patients who have been admitted with an ACS, and to study the temporal pattern of these biomarkers just prior to the occurrence of an imminent repeat coronary event (Oemrawsingh *et al.* 2016). In brief, patients aged $\geq$40 years, who were admitted with an ACS, and who had $\geq$1 cardiovascular risk factor were eligible. The diagnosis ACS was based on typical ischemic chest pain, lasting $>$10 min in the preceding 24 h, in combination with objective evidence of myocardial necrosis, as obtained from the ECG (ST-segment elevation or dynamic ST-segment depression) or biochemistry (CKMB or cardiac troponin elevation). After enrolment, venepunctures were performed every two weeks during the first six months and every four weeks during the second six months of follow-up.

BIOMArCS was approved by the Institutional Review Boards of all 18 enrolling hospitals, and all participating patients provided written informed consent. BIOMArCS is registered in The Netherlands Trial Register NTR1698 and NTR1106.

Table 1. Overview of the immune and inflammatory protein biomarkers.

Abbreviation	Full name	Synonyms	Molecular function
ADAM-TS13	A disintegrin and metalloproteinase with thrombospondin motifs 13	C9orf8, vWF-CP	Hydrolase
ADM	Adrenomedullin	AM	Hormone
ACE2	Angiotensin I converting enzyme 2	Peptidyl-dipeptidase A	Hydrolase/receptor
CXCL1	C-X-C motif chemokine ligand 1	GRO1, GROa, MGSA, FSP, NAP-3	Chemokine/growth factor
CEACAM8	Carcinoembryonic antigen-related cell adhesion molecule 8	CGM6, CD66b	Protein binding
CTSL1	Cathepsin L1		Hydrolase
HO-1	Heme oxygenase (decycling) 1	HMOX1	Oxidoreductase
IL-1ra	Interleukin-1 receptor antagonist	IL1RN, IRAP, ICL-1RA	Cytokine
IL1RL2	Interleukin-1 receptor-like 2	IL1RRP2, IL36R	Receptor
IL-17D	Interleukin-17D	? To do	Cytokine
IL-27	Interleukin-27 subunit alpha and beta	IL27A, EBI3, IL27B	Cytokine
IL-4RA	Interleukin-4 receptor subunit alpha	IL4R	Receptor
LOX-1	Lectin-like oxidized LDL receptor 1	OLR1, CLEC8A	Receptor
LPL	Lipoprotein lipase	LIPD	Hydrolase
IgG Fc receptor II-b	Fc fragment of IgG, low affinity IIb receptor	FCGR2B, CD32B	Receptor
MARCO	Macrophage receptor with collagenous structure	SCARA2	Receptor
hOSCAR	Osteoclast associated, immunoglobulin-like receptor	OSCAR	Receptor
PTX3	Pentraxin 3	TSG14, TNFAIP5	Receptor
PIgR	Polymeric immunoglobulin receptor		Receptor
IL16	Pro-interleukin-16	LCF	Cytokine
PD-L2	Programmed cell death 1 ligand 2	PDCD1LG2, B7DC, CD273	Receptor
RAGE	Advanced glycosylation end product-specific receptor	AGER	Receptor
CD84	SLAM family member 5	SLAMF5	Receptor
SPON2	Spondin-2	Mindin, DIL1	Antigen binding
CD4	T-cell surface glycoprotein CD4		Antigen binding
TF	Coagulation factor III (tissue factor)	F3, thromboplastin	Receptor
TRAIL-R2	TNF-related apoptosis-inducing ligand receptor 2	TNFRSF10B, CD262, DR5	Receptor
TNFRSF10A	Tumour necrosis factor receptor superfamily member 10A	CD261, DR4, TRAILR-1, APO2	Receptor
TNFRSF13B	Tumour necrosis factor receptor superfamily member 13B	CD267, TACI	Receptor

Table 2. Baseline clinical characteristics of the patients.

	Cases $n = 44$	Controls $n = 87$	p Value
Presentation and initial treatment			
Men	35 (79.5)	70 (80.5)	0.90
Age (years)	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)	0.30
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)	0.010
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[a]			
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)	0.10
ACE inhibitor or ARB	34 (89.5)	65 (79.3)	0.17

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

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

Protein (NPX)	Coefficient	95%CI	p Value
ADAM-TS13	0.079	(−0.093 to 0.25)	0.36
ADM	0.36	(0.015 to 0.70)	0.041
ACE2	0.69	(0.18 to 1.20)	0.009
CXCL1	1.00	(0.38 to 1.61)	0.002
CEACAM8	0.38	(−0.070 to 0.83)	0.096
CTSL1	0.21	(−0.10 to 0.52)	0.18
HO-1	0.14	(−0.19 to 0.47)	0.39
IL-1ra	0.11	(−0.49 to 0.71)	0.72
IL1RL2	0.046	(−0.22 to 0.31)	0.73
IL-17D	0.23	(0.029 to 0.43)	0.026
IL-27	0.36	(0.093 to 0.62)	0.009
IL-4RA	0.48	(0.16 to 0.80)	0.004
LOX-1	0.16	(−0.23 to 0.54)	0.42
LPL	−0.12	(−0.42 to 0.17)	0.40
IgG Fc receptor II-b	0.17	(−0.25 to 0.60)	0.42
MARCO	0.086	(−0.069 to 0.24)	0.27
hOSCAR	0.15	(−0.040 to 0.33)	0.12
PTX3	0.34	(−0.13 to 0.80)	0.15
PIgR	0.040	(−0.032 to 0.11)	0.27
IL16	0.21	(−0.14 to 0.56)	0.23
PD-L2	0.21	(−0.010 to 0.42)	0.061
RAGE	0.40	(0.12 to 0.67)	0.006
CD84	0.64	(0.25 to 1.03)	0.002
SPON2	0.14	(0.026 to 0.25)	0.017
CD4	0.19	(−0.024 to 0.41)	0.080
TF	0.14	(−0.049 to 0.33)	0.14
TRAIL-R2	0.29	(−0.041 to 0.62)	0.084
TNFRSF10A	0.41	(0.20 to 0.62)	0.0004
TNFRSF13B	0.096	(−0.23 to 0.43)	0.56

ACS: acute coronary syndrome; CI: confidence interval; NPX: Normalized Protein eXpression.
For every protein biomarker, the difference in 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 serum level.

Case-control design

For the current study, we applied a nested case-control design for protein measurements and statistical analysis (Figure 1). A total of 45 patients (cases) developed the primary study endpoint composed of cardiac death, non-fatal myocardial infarction (MI), or unstable angina (UA) requiring urgent coronary revascularization during one year of follow-up after the initial ACS. Each case was assigned to two controls matched on age, gender and admitted hospital. For reasons of efficiency, for each case, the blood sample at hospital admission and the last two samples prior to the recurrent endpoint event were selected. For matched controls, the blood sample at hospital admission and the blood sample that corresponds in time from enrolment with the timing of the case-event were selected.

We were interested in the temporal patterns of the proteins during the acute phase after the initial ACS (first 30 days), as well as during the stable phase after the initial ACS (30-day to 1-year time period). Thus, separate analyses were conducted for cases (and their matching controls) who experienced the event in the first 30 days of follow-up after the initial ACS, and for cases (and their matching controls) who experienced their event thereafter until one-year follow-up.

Protein measurements

Olink's high throughput Proximity Extension Assay (PEA) technique (Olink Proteomics AB, Uppsala, Sweden) was used to measure 29 immune and inflammatory proteins of the cardiovascular II panel (Table 1) (Assarsson *et al.* 2014). Detailed information on PEA Technique is available on Olink's website (www.olink.com). In brief, PEA technique allows for efficient quantification of multiple protein biomarkers simultaneously. Every measured protein is expressed in an arbitrary unit on the log2-scale called Normalized Protein eXpression (NPX). Hence, an *increase* or *decrease* of *one* NPX corresponds to a *doubling* or *halving* of the protein's serum level, respectively. To determine approximate serum concentrations, general calibrator curves are available on the website of Olink for each protein biomarker.

Statistical analysis

Continuous baseline characteristics are presented as medians with 25th and 75th percentile, and were compared between

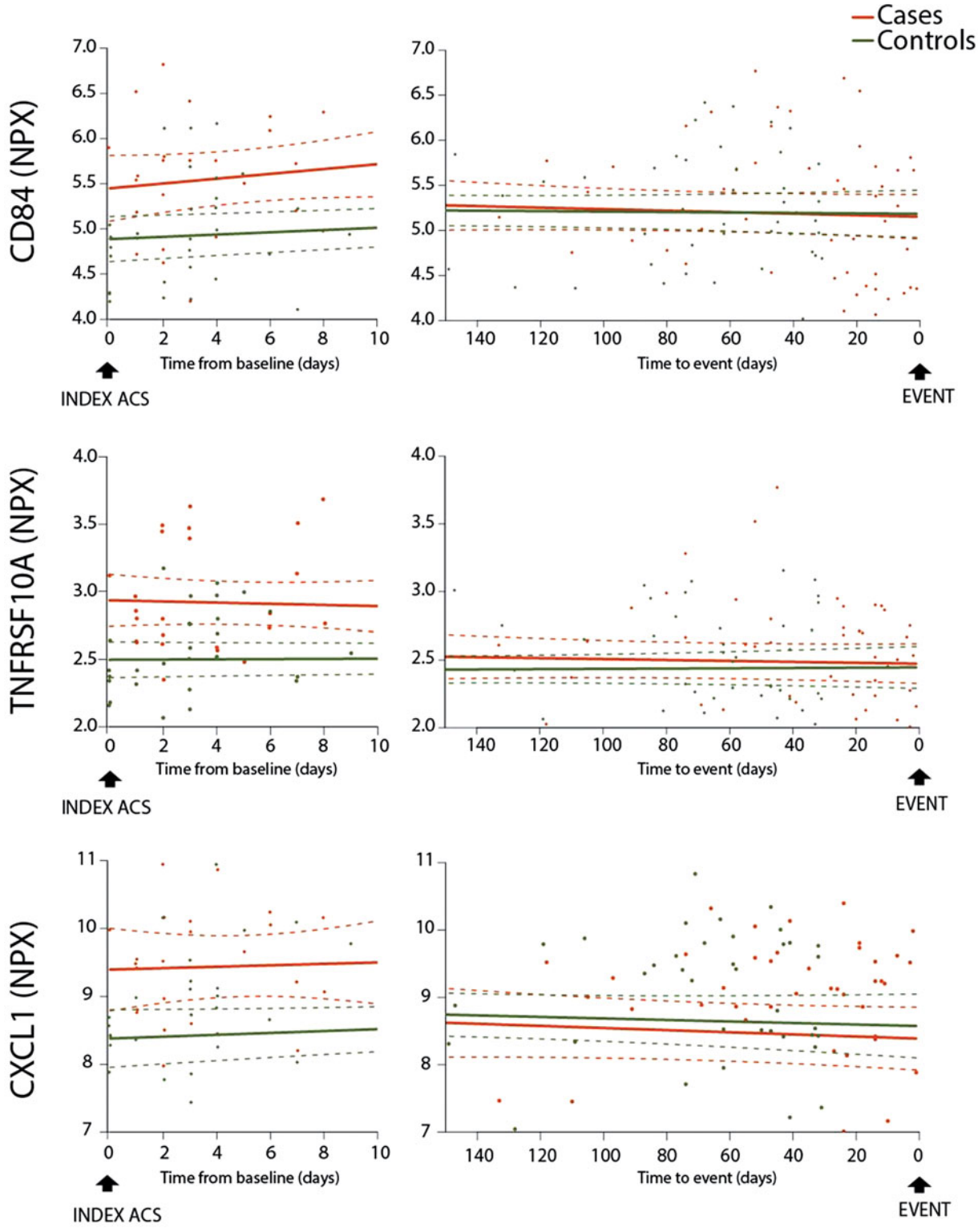

Figure 2. The temporal pattern of CD84, TNFRSF10A and CXCL1. NPX: Normalized Protein eXpression.

cases and controls with the Mann–Whitney *U* test. Categorical baseline characteristics are presented as numbers with percentages and were compared between cases and controls with the Chi-square test.

Linear mixed-effects models were fitted for every protein (dependent). To allow individual variation per patient, random intercepts were included in the models. Likelihood ratio tests and *F* tests were used for hypothesis testing, and model assumptions were checked by visual examination of the residuals. To account for the 29 proteins tested, correction for multiple testing was applied ($p = 0.003$) (Li and Ji 2005). The corrected significance level was calculated using the matrix spectral decomposition method, a correction method used in 'omics' studies to account for the number of independent

Table 4. Protein biomarker serum levels in relation to time post index ACS.

	Median maximum value ≤7 days[a]		Patient-level mean value ≤30 days[b]		Patient-level mean value >30 days[b]	
Protein (NPX)	Cases	Controls	Cases	Controls	Cases	Controls
ADAM-TS13	5.22 (5.02–5.47)	5.19 (4.94–5.31)	5.17 ± 0.32	5.19 ± 0.27	5.20 ± 0.30	5.26 ± 0.31
ADM	7.53 (7.10–8.01)	7.21 (6.75–7.54)	7.36 ± 0.50	7.06 ± 0.55	7.18 ± 0.54	6.99 ± 0.55
ACE2	4.24 (3.95–5.06)	3.95 (3.56–4.29)	4.27 ± 0.92	4.00 ± 0.79	4.21 ± 0.62	4.18 ± 0.86
CXCL1	9.42 (8.76–9.98)	8.68 (7.86–9.22)	8.95 ± 1.07	8.67 ± 1.22	8.46 ± 1.29	8.76 ± 1.22
CEACAM8	3.98 (3.52–4.25)	3.64 (3.09–4.12)	3.76 ± 0.79	3.54 ± 0.65	3.35 ± 0.61	3.30 ± 0.61
CTSL1	5.39 (5.06–5.57)	5.17 (4.85–5.48)	5.30 ± 0.59	5.15 ± 0.49	5.07 ± 0.44	5.02 ± 0.50
HO-1	13.00 (12.63–13.25)	12.79 (12.26–13.20)	12.71 ± 0.50	12.77 ± 0.54	12.55 ± 0.34	12.77 ± 0.52
IL-1ra	7.56 (6.60–8.07)	6.94 (6.44–7.52)	7.11 ± 0.78	7.04 ± 0.92	6.75 ± 0.51	6.81 ± 0.78
IL1RL2	3.13 (2.67–3.47)	3.07 (2.62–3.30)	3.07 ± 0.41	3.07 ± 0.43	3.14 ± 0.34	3.07 ± 0.44
IL-17D	2.49 (2.34–2.71)	2.18 (2.00–2.53)	2.34 ± 0.35	2.22 ± 0.31	2.26 ± 0.22	2.25 ± 0.36
IL-27	3.20 (2.69–3.55)	2.80 (2.51–3.13)	3.05 ± 0.55	2.85 ± 0.42	2.87 ± 0.35	2.79 ± 0.29
IL-4RA	2.21 (1.84–2.91)	1.86 (1.71–2.16)	2.22 ± 0.66	1.88 ± 0.30	1.81 ± 0.26	1.82 ± 0.32
LOX-1	6.33 (5.70–6.96)	5.99 (5.55–6.50)	6.08 ± 0.69	6.03 ± 0.62	5.85 ± 0.49	5.92 ± 0.50
LPL	9.26 (8.67–9.73)	9.09 (8.89–9.67)	9.23 ± 0.50	9.30 ± 0.52	9.40 ± 0.53	9.34 ± 0.49
IgG Fc receptor II-b	3.06 (2.62–3.50)	2.96 (2.43–3.39)	3.01 ± 0.73	2.94 ± 0.70	2.97 ± 0.85	2.88 ± 0.75
MARCO	5.00 (4.79–5.13)	4.93 (4.75–5.19)	4.98 ± 0.22	4.98 ± 0.29	4.99 ± 0.26	5.02 ± 0.29
hOSCAR	10.60 (10.42–10.78)	10.43 (10.19–10.77)	10.58 ± 0.30	10.42 ± 0.35	10.51 ± 0.31	10.40 ± 0.35
PTX3	3.96 (2.96–4.47)	3.38 (2.98–4.06)	3.43 ± 0.82	3.31 ± 0.68	3.03 ± 0.56	2.91 ± 0.51
PIgR	7.25 (7.10–7.33)	7.17 (7.07–7.27)	7.18 ± 0.17	7.17 ± 0.14	7.20 ± 0.18	7.19 ± 0.15
IL16	5.41 (5.18–5.82)	5.23 (4.85–5.54)	5.30 ± 0.48	5.18 ± 0.60	5.33 ± 0.37	5.15 ± 0.52
PD-L2	2.64 (2.30–3.06)	2.44 (2.16–2.74)	2.64 ± 0.44	2.50 ± 0.38	2.65 ± 0.48	2.60 ± 0.37
RAGE	5.57 (5.31–5.92)	5.30 (4.98–5.51)	5.45 ± 0.50	5.14 ± 0.39	5.30 ± 0.51	5.13 ± 0.43
CD84	5.54 (4.86–5.80)	4.93 (4.53–5.33)	5.34 ± 0.62	5.09 ± 0.63	5.17 ± 0.67	5.24 ± 0.65
SPON2	10.28 (10.11–10.43)	10.13 (9.95–10.26)	10.18 ± 0.26	10.09 ± 0.24	10.17 ± 0.21	10.11 ± 0.26
CD4	2.99 (2.48–3.25)	2.74 (2.45–3.02)	2.83 ± 0.38	2.79 ± 0.46	2.87 ± 0.40	2.85 ± 0.46
TF	5.60 (5.48–5.85)	5.55 (5.35–5.78)	5.55 ± 0.31	5.54 ± 0.32	5.59 ± 0.37	5.58 ± 0.36
TRAIL-R2	5.79 (5.48–6.42)	5.65 (5.34–5.93)	5.69 ± 0.60	5.60 ± 1.04	5.61 ± 0.67	5.47 ± 1.03
TNFRSF10A	2.80 (2.50–3.32)	2.48 (2.15–2.78)	2.69 ± 0.44	2.46 ± 0.40	2.50 ± 0.39	2.45 ± 0.36
TNFRSF13B	7.47 (7.33–7.86)	7.55 (7.05–7.85)	7.72 ± 0.88	7.60 ± 0.54	7.89 ± 1.05	7.71 ± 0.59

NPX: Normalized Protein eXpression.
Blood samples in the time windows 0–7, 8–30 and 30–365 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.

performed tests (Nyholt 2004, Li and Ji 2005). All statistical tests were two-tailed. R statistical software (version 3.4.0) was used for statistical analyses, in particular the package nlme (https://cran.r-project.org/web/packages/nlme/index.html).

Results

Baseline characteristics

Mean age was 66 ± 12 years and 80% were men. Presentation, initial treatment, cardiovascular risk factors and medication at first blood sample (baseline) were similar for cases and matched controls (Table 2). Thus, matching was successful.

Biomarker pattern within first 30 days post-ACS

Fifteen cases experienced a recurrent event within the first 30 days of follow-up. After correction for multiple testing, the serum level of CXCL1 in the first 30 days was 1.00 NPX (95% confidence interval (CI) 0.38–1.61) higher in cases than in their matching controls, which corresponds to a doubling of the CXCL1 serum level in cases. The serum levels of CD84 and TNFRSF10A were also significantly higher in cases than in their matching controls with a difference in these serum levels of 0.64 NPX (95%CI 0.25–1.03) and 0.41 NPX (95%CI 0.20–0.62), respectively (Table 3, Figure 2 left-hand panel).

Biomarker pattern after 30 days

Twenty nine cases experienced their recurrent event between 30 days and one year following their initial ACS. Prior to the recurrent coronary event in the 30-day to one-year period, serum levels of all studied protein biomarkers stabilized in cases and matched controls to indistinctive serum levels (Table 4). Hence, we found no significant divergent protein biomarker patterns between so-called late cases and matched controls. In particular, no (steady) increase was observed prior to the repeat event.

Since we did find significant divergent protein biomarker patterns between early cases and matched controls, we compared protein biomarker serum levels of ≤30-day cases with those of >30-day cases as a post hoc analysis in the first 30 days post index-ACS (Table 5). Overall, most protein biomarker serum levels appeared to be higher in early cases.

Discussion

This study assessed the temporal pattern of 29 immune and inflammatory proteins in post-ACS patients. Serum levels of CXCL1, CD84 and TNFRSF10A showed to be significantly higher in cases than in matched controls prior to their recurrent coronary event within 30 days after the index ACS. After the first 30 days of follow-up, none of the studied protein biomarkers had detectable divergent serum levels in cases and their matched controls.

Table 5. Protein biomarker serum levels in the first 30 days for cases only.

Protein (NPX)	Early cases[a]	Late cases[a]	*p* Value
ADAM-TS13	5.25 ± 0.30	5.10 ± 0.34	0.20
ADM	7.53 ± 0.50	7.21 ± 0.45	0.067
ACE2	4.64 ± 1.03	3.94 ± 0.70	0.032
CXCL1	9.45 ± 0.69	8.51 ± 1.16	0.010
CEACAM8	3.98 ± 0.72	3.56 ± 0.82	0.13
CTSL1	5.42 ± 0.59	5.20 ± 0.59	0.30
HO-1	12.91 ± 0.49	12.53 ± 0.45	0.028
IL-1ra	7.23 ± 0.84	7.00 ± 0.73	0.42
IL1RL2	2.99 ± 0.43	3.14 ± 0.40	0.33
IL-17D	2.48 ± 0.37	2.22 ± 0.28	0.032
IL-27	3.26 ± 0.59	2.87 ± 0.45	0.045
IL-4RA	2.44 ± 0.75	2.03 ± 0.51	0.079
LOX-1	6.11 ± 0.48	6.06 ± 0.84	0.83
LPL	9.20 ± 0.51	9.25 ± 0.51	0.77
IgG Fc receptor II-b	2.97 ± 0.74	3.05 ± 0.74	0.77
MARCO	5.02 ± 0.21	4.95 ± 0.23	0.36
hOSCAR	10.62 ± 0.23	10.54 ± 0.35	0.48
PTX3	3.64 ± 0.93	3.24 ± 0.68	0.18
PIgR	7.23 ± 0.11	7.14 ± 0.20	0.10
IL16	5.34 ± 0.42	5.27 ± 0.54	0.68
PD-L2	2.70 ± 0.35	2.58 ± 0.51	0.45
RAGE	5.65 ± 0.47	5.28 ± 0.47	0.036
CD84	5.58 ± 0.63	5.13 ± 0.55	0.039
SPON2	10.28 ± 0.21	10.08 ± 0.27	0.031
CD4	2.95 ± 0.33	2.72 ± 0.39	0.079
TF	5.69 ± 0.24	5.43 ± 0.31	0.011
TRAIL-R2	5.88 ± 0.64	5.53 ± 0.53	0.10
TNFRSF10A	2.92 ± 0.41	2.48 ± 0.36	0.003
TNFRSF13B	7.66 ± 0.40	7.78 ± 1.17	0.71

NPX: Normalized Protein eXpression.
Blood samples ≤30 days after the index ACS were available for 15 early cases and 17 late cases.
[a]Patient-level mean value ± standard deviation.

CXCL1 is a cytokine that attracts neutrophils by chemotaxis and stimulates monocyte arrest (Dusi *et al.* 2016). Oxidized LDL and wall shear stress on endothelial cells have been shown to induce the expression of CXCL1 (Hagiwara *et al.* 1998, Zhou *et al.* 2011). Subsequently, CXCL1 stimulates monocyte adhesion to the endothelial wall (Breland *et al.* 2008, Papadopoulou *et al.* 2008). These monocytes migrate into the endothelial wall and stimulate the accumulation of macrophages. Eventually, this process promotes atherosclerotic plaque formation and instability and is therefore a key process in pathological atherosclerosis. Since we found higher serum levels of CXCL1 in early cases, a possible mechanism may be that CXCL1 is upregulated due to the index ACS, but subsequently also promotes early recurrent events by inducing atherosclerotic plaque instability.

CD84 is a signalling lymphocyte activation molecule (SLAM) and is expressed on platelets. It has been described that during thrombus formation, CD84 is triggered upon platelet aggregation and advances thrombus stability (Nanda *et al.* 2005). Since disproportional thrombus formation may cause arterial occlusion, CD84 may be of interest as a biomarker for coronary events. However, little research has been conducted on CD84 and its association with CVD. One previous study has identified CD84 to be associated with adverse outcome in Kawasaki disease coronary arteriopathy (Reindel *et al.* 2014). In this study, CD84 was found to be highly expressed in inflamed endothelium tissue of the coronary arteries of patients who developed adverse outcome and was suggested to play an important role in the pathogenesis of arterial thrombosis (Reindel *et al.* 2014). Our study found higher CD84 serum levels in post-ACS patients who developed early recurrent events. Potentially, CD84 upregulation is initiated by the index ACS and, subsequently, promotes disproportional thrombus formation causing early recurrent ACS. Further research should establish whether CD84 serum levels may be used to identify patients who will develop an early recurrent coronary event and who will not.

TNFRSF10A, a receptor for TNFSF10/TRAIL, is a member of the TNF-receptor superfamily and modulates apoptosis and proliferation of vascular smooth muscle cells (Pan *et al.* 1997, Kavurma *et al.* 2008). Since these processes may be beneficial as well as disadvantageous for atherosclerosis, depending on the stage of an atherosclerotic lesion, there is still an ongoing debate as to whether TNFRSF10A and its ligand may be used as a marker for progression or regression of atherosclerosis (Forde *et al.* 2016). Our study found higher serum levels of TNFRSF10A in patients who developed an early recurrent cardiac event. Potentially, higher TNFSRF10A serum levels induce excessive proliferation of vascular smooth muscle cells after the index ACS which may lead to new coronary stenosis (Kavurma and Bennett 2008).

Our study shows that CXCL1, CD84 and TNFRSF10A serum levels were elevated in post-ACS patients who experienced an early repeat coronary event. Nonetheless, we did not find divergent protein biomarker serum levels in post-ACS patients who experienced a late repeat coronary event (i.e. in the 30-day to one-year time-window). One may argue that differences in serum levels between cases and controls may be smaller in the long-term and our study lacked power to reveal these. We designed the current study as an initial analysis and did not quantify all collected blood samples in BIOMArCS, since we intended – depending on the first results – to assess more blood samples after our first analysis to expand the number of repeated biomarker measurements. However, since we did find associations in early cases, and our study did not lack power to reveal these associations, we conclude that the 29 protein biomarkers we studied may not be associated with the development of recurrent coronary events in late cases. Apparently, the index ACS triggers short-term upregulation of CXCL1, CD84 and TNFRSF10A, which may play a role in the development of early recurrent coronary events.

For our protein measurements, we used Olink's PEA technique. This PEA technique enables an effective assessment of blood samples with a rapid high-throughput analysis of high sensitivity and specificity. However, although PEA is a promising technique, improvements are warranted to assure clinical valid and reproducible measurements. In addition to the technical challenges, one should consider that other factors related to biobank-sampling, i.e. blood sample collection and repeated freezing and thawing of collected blood samples influence the reproducibility of protein measurements. Studying the behaviour over time of immune and inflammatory proteins in patients with CVD prior to a (recurrent) coronary event may provide new insights on modulators of pathological atherosclerosis. However, current research remains exploratory. Technical improvements are required to assess whether immune and inflammatory proteins can be

used in clinical practice and may contribute to established clinical tools for disease detection and prognosis. Finally, the proposed mechanisms through which the biomarkers may be pathophysiologically related to the repeat ACS are hypothesis generating.

Conclusions

Among 29 immune and inflammatory proteins on the Olink platform, CXCL1, CD84 and TNFRSF10A were associated with early repeat coronary events in patients who experienced an ACS. Further research should assess whether CXCL1, CD84 and TNFRSF10A can actually be used to discriminate between patients who will experience an early repeat coronary event after an initial ACS admission, and patients who will not.

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