Towards identification of novel putative biomarkers for infective endocarditis by serum proteomic analysis

Snipsøyr, Magnus Giske; Wiggers, Henrik; Ludvigsen, Maja; Stensballe, Allan; Vorum, Henrik; Poulsen, Steen Hvitfeldt; Rasmussen, Lars Melholt; Petersen, Eskild; Honoré, Bent

Published in:
International Journal of Infectious Diseases

DOI (link to publication from Publisher):
10.1016/j.ijid.2020.02.026

Publication date:
2020

Document Version
Publisher's PDF, also known as Version of record

International Journal of Infectious Diseases 96 (2020) 73–81

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Towards identification of novel putative biomarkers for infective endocarditis by serum proteomic analysis

Magnus Giske Snipsøyr[a,b,c], Henrik Wiggers[b], Maja Ludvigsen[a], Allan Stensballe[d], Henrik Vorum[e], Steen Hvitfeldt Poulsen[b], Lars Melholt Rasmussen[f], Eskild Petersen[c], Bent Honoré[a,e,*]

[a] Department of Biomedicine, Aarhus University, Aarhus, Denmark
[b] Department of Cardiology, Aarhus University Hospital, Skejby, Denmark
[c] Department of Infectious Diseases, Aarhus University Hospital, Skejby, Denmark
[d] Department of Health Science and Technology, Aalborg University, Denmark
[e] Department of Clinical Medicine, Aalborg University, Denmark
[f] Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Odense, Denmark

ARTICLE INFO

Article history:
Received 28 November 2019
Received in revised form 13 February 2020
Accepted 14 February 2020

Keywords:
Biomarkers
Infective endocarditis
LFQ LC-MS/MS
Non-infective endocarditis
Serum proteomics
2D-PAGE

ABSTRACT

Introduction: Infective endocarditis (IE) has high mortality, partly due to delayed diagnosis. No biomarker can identify IE in patients with fever and clinical picture of infection. To find putative biomarkers we analyzed serum levels of two proteins found in cardiac valves, fibulin-1 ($n = 696$) and osteoprotegerin ($n = 689$) among patients on clinical suspicion of IE. Proteomic analyses were performed in 24 patients with bacteremia, 12 patients with definite IE and 12 patients with excluded IE.
Methods: Fibulin-1 and osteoprotegerin were studied by enzyme linked immunosorbent assay (ELISA). Proteomic analyses were conducted by 2-dimensional polyacrylamid gel electrophoresis (2D-PAGE) and label-free quantitative liquid chromatography - tandem mass spectrometry (LFQ LC-MS/MS). Controls for 2D 2D-PAGE and LFQ LC-MS/MS had bacteremia and excluded IE.
Results: Osteoprotegerin levels were significantly increased in IE patients compared with non-IE patients. Fibulin-1 showed no difference. 2D-PAGE showed significant differences of 6 proteoforms: haptoglobin, haptoglobin-related protein, α-2-macroglobulin, apolipoprotein A-I and ficolin-3. LFQ LC-MS/MS analysis revealed significant level changes of 7 proteins: apolipoprotein L1, complement C1q subcomponent B and C, leukocyte immunoglobulin-like receptor subfamily A member 3, neuropilin-2, multimerin-1 and adiponectin.
Conclusions: The concentration changes in a set of proteoforms/proteins suggest that stress and inflammation responses are perturbed in patients with IE compared to patients with bacteremia without IE.

Introduction

The incidence of infective endocarditis (IE) has increased during the last decades to 1.7–6.2 cases per 100,000 population years (Beynon et al., 2006; Bouza et al., 2001; Delahaye et al., 1995; Griffin et al., 1985; Mylonakis and Calderwood, 2001). IE has a high mortality, and despite treatment, the 12 month mortality reached 30% (Thuny et al., 2012a). The clinical challenge is to determine whether a febrile patient with either a positive blood culture or a suspected bacteremia also has IE. The diagnostic delay of IE can be several weeks (Issa et al., 2003; Knudsen et al., 2009), increasing the risk of death, heart failure, valve replacement and embolic complications. The key diagnostic procedures are blood cultures and transthoracic and transesophageal echocardiography. Biomarkers aiding in the evaluation of patients with a low- or intermediate risk of endocarditis would therefore be of great clinical value.

At present, there are no biofluid biomarkers, which are used in clinical practice to identify IE in patients with infection and/or bacteremia (Snipsøyr et al., 2016). One problem with previous studies has been the lack of proper control groups, i.e. patients

* Corresponding author at: Department of Biomedicine, Aarhus University, Aarhus, Denmark, Ole Worms Allé 4, 8000 Aarhus C, Denmark.
E-mail address: bh@biomed.au.dk (B. Honoré).

https://doi.org/10.1016/j.ijid.2020.02.026

with bacteremia and excluded endocarditis (Snipsøyr et al., 2016). C-reactive protein (CRP) is a common biomarker for inflammation but lacks sensitivity and specificity to distinguish between patients with positive blood cultures with or without IE. Procalcitonin has been suggested to differentiate between patients with and without endocarditis (Mueller et al., 2004). However, data from our group have shown that although procalcitonin is significantly higher in IE patients than non-IE patients, it has a low specificity (Knudsen et al., 2010).

The aim of the present study was to identify biomarkers of IE in two ways. First, we investigated the potential of fibulin-1 and osteoprotegerin (OPG) for the diagnosis of IE in a group of patients suspected for IE. Fibulin-1 and OPG are markers of cardiovascular disease (Hansen et al., 2013; Jono et al., 2002; Kruger et al., 2014; Mogelvang et al., 2012; Palazzuoli et al., 2008; Ren et al., 2008; Schoppet et al., 2003). Fibulin-1 is a matrix protein and therefore also a structural part of the heart valves (Argraves et al., 2009). OPG has been reported to be a mediator of the calcification development of aortic stenosis (Dahl et al., 2013), as well as possibly being involved in the pathological processes and being a marker of mortality in atrial fibrillation (Ueland et al., 2011). Second, we identified putative biomarkers by proteomic investigation using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) as well as label-free quantitative liquid chromatography – tandem mass spectrometry (LFQ LC-MS/MS) on serum samples from IE and non-IE patients. We hypothesized that these proteomics methods could detect proteins released from cardiac valve tissue, inflammatory proteins or blood coagulation proteins in order to find new putative markers of IE. In the proteomic analyses we used a control group of patients with bacteremia and with endocarditis excluded.

Materials and methods

Patients

The patients studied here were included in a previous study of 759 consecutive patients referred for echocardiographic examination on clinical suspicion of IE (Knudsen et al., 2010). Patients underwent transthoracic and transesophageal echocardiography and a 10 mL blood sample was obtained. Samples were centrifuged, separated and frozen within 4 h. According to the Duke criteria (Durack et al., 1994; Li et al., 2000) 112 patients had definite IE, 35 patients possible IE and 612 patients no IE. Among the patients having definite and possible IE 118 (80%) had positive blood culture while among those excluded for having IE 145 (24%) had positive blood culture (Knudsen et al., 2010).

Among the 759 patients in the previous report, 63 patients had insufficient serum for further analysis. Of the 696 patients included here, 102 had definite IE, 31 had probable IE, while 563 had no IE. The characteristics of the 696 patients included here are shown in the Supplementary Table 1. Following fibulin-1 measurements, 7 patients lacked material for further analyzes, resulting in a total of 689 patients with OPG-measurements.

2D-PAGE and MS for protein identification were performed in a subset of 12 patients with definite IE and damaged heart valves verified by transesophageal echocardiography (TEE) and 12 matched patients with bacteremia and excluded IE diagnosis. The characteristics are shown in the Supplementaty Table 2. All 12 patients in the study group as well as the 12 patients in the control group had bacteremia. Patients are matched with respect to age, gender, microbiological agent (4 patients with Staphylococcus aureus, 2 patients with Enterococcus faecium and 6 patients with Streptococcus species), CRP and hemoglobin measurement.

ELISA for measurement of fibulin-1 and OPG

In all patients with sufficient amount of serum, we measured fibulin-1 ($n = 696$) and OPG ($n = 689$) by established ELISA as described previously (Cangemi et al., 2011; Hansen et al., 2013). Differences between groups of patients were calculated using a non-parametrical, two-tailed Mann-Whitney *U*-test. A $p < 0.05$ was considered significant.

Preparation of serum samples for proteomics

High abundant protein levels in serum is a large problem for proteomic analysis since they may mask lower abundant proteins (Farrah et al., 2011). We used two approaches to try to circumvent this, immunodepletion of high abundant proteins and hexapeptide enrichment of low abundant proteins. Proteome Purify™ 12 immunodepletion resin (R&D Systems, Minneapolis, MN, USA) was used to deplete serum for 12 high abundant proteins, essentially as described by the manufacturer. To 30 μL of serum was added 1 mL of suspended immunodepletion resin and left for 30-60 minutes. on a rotary shaker. The resin was applied to Spin-X filter units and centrifuged for 2 min. The proteins in the filtrate were then precipitated using 5×vol. of 100% acetone at −20 °C overnight. Samples were then centrifuged, washed with 50% acetone, dried and dissolved in 2D-PAGE lysis buffer (9 M urea, 2% (v/v) Triton X-100, 2% (v/v) immobilized pH gradient (IPG) buffer (pH 4-7), 2% (w/v) dithiotreitol (DTT)). Rehydraton buffer (8 M urea, 2% (wt/vol) CHAPS, 2% (vol/vol) IPG-buffer and 0.3% (wt/vol) DTT) was added and the amount applied to the 2D gels was 200 μg. For the ProteoMiner™ Protein Enrichment Kit (BioRad, Hercules, CA, USA) 1 mL of serum samples were incubated with the beads as described and finally eluted with rehydrated elution reagent (8 M urea, 2% CHAPS, 5% acetic acid). Proteins were acetone precipitated as above and dissolved in 2D-PAGE lysis buffer, rehydration buffer was added and an amount of 250 μg was used for 2D-PAGE. For LFQ LC-MS/MS 30 μg protein was mixed with reducing sample buffer and separated by 1D-PAGE (Biorad TGX 4-16%). The electrophoresis was stopped after migration of proteins approx. 1 cm into the gel visualized by CBB. The gel plugs were excised and samples were reduced at 60 °C using 10 mM DTT for 30 min and alkylated using 55 mM iodoacetamide for 30 min. Two μg trypsin (Promega) was added to each gel sample and incubated overnight. The peptides were extracted by 50% acetronitrile and 0.1% formic acid followed by vacuum evaporation. The samples were reconstituted in 2% acetonitrile in 0.1% formic acid.

Protein separation by 2D-PAGE and identification by LC-MS/MS

The 2D-PAGE method has previously been described (Kamper et al., 2011). In the first dimension, proteins were separated according to their isoelectric point and in the second dimension according to molecular mass. Proteins were stained using Sypro-Ruby, where after they were visualized by ImageQuant LAS4010 (GE Healthcare, Buckinghamshire, UK). Subsequently, scanned gel pictures were imported into and analyzed with PDQuest (BioRad, Hercules, CA, USA) software for the Proteome Purify™ analysis, and Delta2D (DECODON, Greifswald, Germany) software for ProteoMiner™ samples. The intensity of each spot was normalized to the total intensity detected in the gel. Spots were matched between gels and the mean relative intensity of each spot in each group was calculated. The mean relative intensity of a spot in the IE group divided by the mean relative intensity of the spot in the non-IE group is listed as the fold change. Relative intensity changes of at least 1.3-fold that were significant ($p < 0.05$, Mann–Whitney, *U*-test) between the groups were detected. Finally, the spots were cut out of the gel for protein identification as described previously

(Kamper et al., 2011). After in-gel trypsinization overnight, proteins were pretreated using acetonitrile and reduced with dithiothreitol. Thereafter, iodoactemide was used to modify cysteine residues in the proteins prior to cleavage with trypsin. Eventually, peptide extraction was performed using one change of Na_4HCO_3 and 3 changes of formic acid in acetonitrile. The recovered proteins were then dried and subsequently dissolved in 12 μL of buffer A (97.7% H_20, 2% acetonitrile, and 0.3% formic acid). Peptides were separated by nanoLC using an Easy nLC II (Thermo Fisher Scientific, Waltham, MA, USA). MS/MS-analysis was performed on a Q-TOF Premier (Waters, Milford, MA, USA). With a flow rate of 300 nL/min, [Glu1]-Fibrinopeptide B (GFP), 300 fmol/L was used as lock mass. GFP was also used for calibration. Masslynx v4.0 (Waters, Milford, MA, USA) was used to acquire MS survey scans in the m/z-interval ranging from 450–1500. MS/MS scans were acquired in the m/z-interval 50–1800. Eventually, raw data were imported into ProteinLynx Global Server v2.3 (Waters, Milford, MA, USA) and the data was used to search the Swiss-Prot Database with the online version of the Mascot MS/MS Ion Search facility (Matrix Science, Ltd., Boston, MA, USA, http://www.matrixscience.com) (Perkins et al., 1999). These searches were conducted with the following criteria: Doubly, triply and quadruply charged ions with up to two missed cleavages, peptide mass tolerance of 20 ppm, an MS/MS tolerance of 0.05 Da, and one fixed modification, Carbamidomethyl (C). Peptides that were considered as contaminants (cingulin, hornerin, filaggrin-2, BSA, trypsin and keratins) were excluded, along with cross contaminated peptides from previous samples. We manually assessed spectra of uncertain identifications and excluded those with bad quality. To qualify as a protein hit, there had to be a minimum of one significant peptide identified ($p < 0.05$).

Direct protein identification and quantification by LFQ LC-MS/MS

Each sample was injected in duplicate into a Dionex RSLC nanoUPLC system that was connected to a Quadrupole Orbitrap (Q ExactivePlus) mass spectrometer equipped with a NanoSpray Flex ion source (Thermo Scientific, Bremen, Germany). The flow settings were 8 μL per min. for the sample loading onto a trapping column, which was an Acclaim PepMap100 C18, 5 μm column from Thermo Scientific. The nanoflow was set to 300 nL per min for the peptide separation on the analytical column, which was a 50 cm Acclaim Pepmap RSLC, 75 μm inner diameter column connected with nanoviper fittings. The nano-electrospray was done using a Picotip 'Silicatip' emitter from New Objective. The LC buffers were buffer A (99.9% water, 0.1% formic acid) and buffer B (99.9% acetonitrile, 0.1% formic acid). The applied gradient was from 10 to 45% buffer B over 35 min. The mass spectrometer was operated in data-dependent acquisition mode. A full MS scan in the mass range of 350 to 1850 m/z was acquired at a resolution of 70,000 with an AGC target of 3×10^6 and maximum fill time set to 250 ms. Instrument lock mass correction was applied using the contaminant ion at 391.28429 m/z. In each cycle, the mass spectrometer will trigger up to 12 MS/MS acquisitions on abundant peptide precursor ions. The MS/MS scans were acquired with a dynamic mass range at a resolution of 17,500, and with an AGC target of 2×10^5 and max fill time of 60 ms. The precursor ions were isolated using a quadrupole isolation window of 1.6 m/z and then fragmented in the HCD trap with a normalized collision energy set to 30. The under-fill ratio was set to 3.5% with the intensity threshold at 1.2×10^5. Apex triggering was 3 to 10 s with charge and exclude isotopes exclusion on, and dynamic exclusion set to 30 s. Protein identification and quantification were done with MaxQuant (v 1.5.5.1) (Tyanova et al., 2016a). The reviewed human Swiss-Prot sequence database was downloaded on the 25th of August 2016 from Uniprot. The label free quantification (LFQ) algorithm was activated. False discovery rates for PSM, protein and site were each set at 1%. The LFQ minimum ratio count was set at 1. MS/MS was required for LFQ comparisons. The match between runs function was activated. Protein quantifications were performed using unique and razor peptides, modified with oxidation (M) or acetyl (protein N-terminal). Reversed sequences as decoys and contaminant sequences have been added automatically by MaxQuant. The results from MaxQuant were entered into Perseus (v 1.5.4.1) (Tyanova et al., 2016b) where data was filtered and Log_2 transformed. The protein amount in a sample was calculated from the means of two technical replicates based on the normalized peptide intensities belonging to the specific protein group. The fold-changes of protein groups were calculated by taking the mean in the IE group divided by the mean in the non-IE group. The analysis was performed in steps requiring the proteins to be detected in at least 70%, 80%, 90% and 100% of the samples. *P*-values were calculated by a two-tailed *t*-test without further correction in order not to increase the type 2 error with the risk of overlooking putative markers.

Bioinformatic analysis

STRING version 11.0 (string-db.org) was used for bioinformatic analysis (Szklarczyk et al., 2015) with the following proteins: HPT_HUMAN (Haptoglobin), HPTR_HUMAN (Haptoglobin-related protein), APOA1_HUMAN (Apolipoprotein A-I), A2MG_HUMAN (Alpha-2-macroglobulin), FCN3_HUMAN (Ficolin-3), APOL1_HUMAN (Apolipoprotein L1), C1QB_HUMAN (Complement C1q subcomponent subunit B), C1QC_HUMAN (complement C1q subcomponent subunit C), NRP2_HUMAN (Neuropilin-2), MMRN1_HUMAN (Multimerin-1), ADIPO_HUMAN (Adiponectin), TR11B_HUMAN (OPG). LIRA3_HUMAN was not recognized by STRING.

Results

Fibulin-1

The ELISA analysis for fibulin-1 did not show any significant differences between groups investigated (data are given in the Supplementary Figure 1). Thus, it seems that this biomarker has no role in the diagnosis of IE.

Osteoprotegerin (OPG)

OPG was measured in all patients with sufficient serum available, in total 689 patients (Table 1). OPG was significantly increased in serum from IE-patients ($n = 132$) compared to the control group of patients without IE ($n = 557$) (median 2.40 ng/mL (range 1.67–3.21) vs. 1.92 ng/mL (1.38–2.90), respectively, $p = 0.002$) (Table 1 and Supplementary Figure 2). When analyzing patients with definite IE according to the Duke Criteria ($n = 101$), we found a highly significant difference (2.57 ng/mL (1.82–3.38) vs. 1.92 ng/mL (1.38–2.90), $p = 0.0008$).

Table 1
OPG serum levels (ng/mL) measured in 689 patients belonging to different groups.[a]

Group (n)[b]	Median (IQR)[c] (ng/mL)	p-Value
Non-IE patients (557)	1.92 (1.38–2.90)	
IE-patients (132)	2.40 (1.67–3.21)	0.002
Definite IE[d] (101)	2.57 (1.82–3.38)	0.0008

[a] All groups are compared to the non-IE group.
[b] n = number of patients.
[c] IQR = Interquartile range.
[d] According to the Duke Criteria.

A *receiver operating curve* (ROC) analysis for patients with confirmed IE according to the Duke Criteria compared to the non-IE group gave an *area under the curve* (AUC) equal to 0.605 (95% confidence interval [CI], 0.54–0.67). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for different cut-off values are given in the Supplementary Table 3. When comparing all IE patients with the non-IE group, the AUC was 0.588.

Proteomic analysis

High abundant proteins in serum is a large problem for analysis since they may mask lower abundant proteins (Farrah et al., 2011). With the 2D-PAGE technique we used two different preparation techniques in order to deplete the serum samples for high abundant proteins. One used Proteome Purify™ and the other used ProteoMiner™. In each case at least 550 spots were detected. Figure 1 shows a representative sample immunodepleted for 12 proteins of high abundance using Proteome Purify™ and Figure 2 shows a representative sample depleted for high abundant proteins and enriched for low abundant proteins using ProteoMiner™. Thirteen protein spots differed significantly ($p < 0.05$) at least 1.3-fold. Two spots were upregulated and eleven were downregulated. The spots were excised from the gels and subjected to LC-MS/MS identification as given in Table 2. Some spots contained more than one protein identification, in which case we cannot determine with certainty which protein is responsible for the observed change. There were no upregulated spots with only one identified protein. Six downregulated spots with only one identified protein were found. They were haptoglobin (2203), haptoglobin-related protein (2306), apolipoprotein A-I (4004) and α-2-macroglobulin (5905 and 5910) shown in Figure 1 and ficolin-3 (445) shown in Figure 2. Spot numbers and fold changes are listed in Table 2.

With the LFQ LC-MS/MS method we used the ProteoMiner™ kit and detected 532 different protein groups combined in all samples. Three hundred eleven were present in at least 70% of all samples in each group, and amongst these we found 7 to be differentially regulated as given in Figure 3 and Table 3. Significantly upregulated protein groups were apolipoprotein L1 (APOL1), complement C1q subcomponent subunits B and C (C1QB, C1QC) and leukocyte immunoglobulin-like receptor subfamily A member 3 (LILRA3) while neuropilin-2 (NRP2), multimerin-1 (MMRN1) and adiponectin (ADIPOQ) were downregulated. Fibulin-1 did not show any significant differences between the groups in the proteomic analysis in line with the ELISA measurements (not shown).

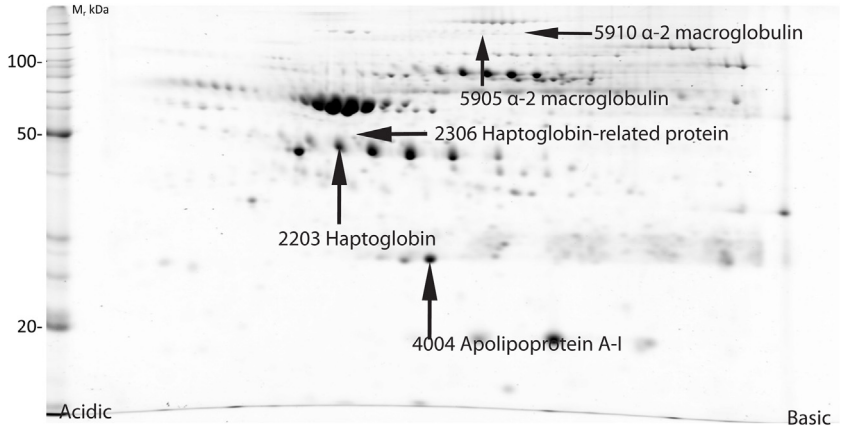

Figure 1. 2D-gel analysis showing differentially expressed protein spots between the IE group and the control group (non-IE patients with bacteremia) based on the Proteome Purify™ 12 immunodepletion resin. All spots differing significantly ($p < 0.05$) at least 1.3-fold between the groups and containing one identified protein are marked with arrows and spot number. The identifications (haptoglobin, haptoglobin-related protein, α-2-macroglobulin (2 proteoforms), apolipoprotein A–I) are listed in Table 2.

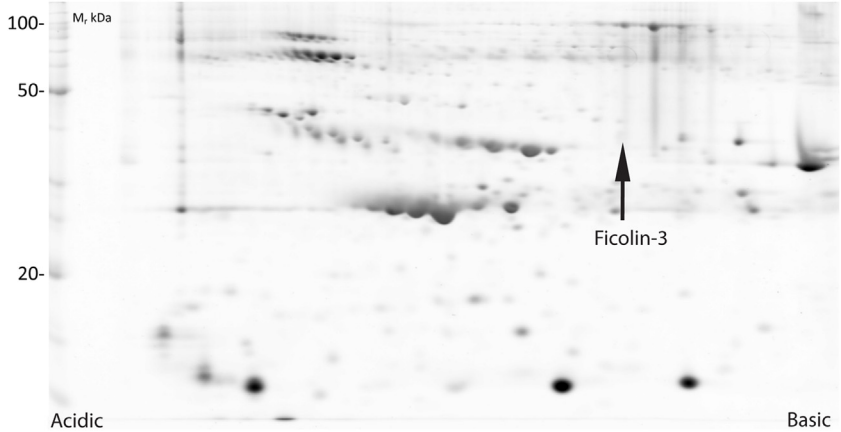

Figure 2. 2D-gel analysis showing differentially expressed protein spots between the IE group and the control group (non-IE patients with bacteremia) based on the ProteoMiner™ Protein Enrichment Kit. One spot differing significantly ($p < 0.05$) at least 1.3-fold between the groups and containing one identified protein is marked with arrow and spot number. The identification (ficolin-3) is listed in Table 2.

Table 2
Identification of differentially expressed proteins (proteoforms) in patients with IE vs. non-IE by 2D-PAGE

SSP	Identification	Peptides	Mascot Score	Mr (Da)	SwissProt	Fold change (IE/non-IE)[a]	*p*-Value
1207	Haptoglobin	4	284	45,177	HPT_HUMAN	0.53	0.045
	Haptoglobin	1	82	38,457	HPT_PIG		
	Complement C4-A	2	134	192,664	CO4A_HUMAN		
2203	Haptoglobin	8	510	45,177	HPT_HUMAN	0.51	0.033
	Haptoglobin	1	84	38,457	HPT_PIG		
	Haptoglobin	1	57	38,577	HPT_MESAU		
2306	Haptoglobin-related protein	1	58	39,005	HPTR_HUMAN	0.51	0.045
2502	Alpha-1-antitrypsin	3	184	46,707	A1AT_HUMAN	4.32	0.0032
	Angiotensinogen	2	157	53,152	ANGT_HUMAN		
	Ig gamma-1 chain C region	1	69	36,083	IGHG1_HUMAN		
	Haptoglobin-related protein	1	58	39,005	HPTR_HUMAN		
4004	Apolipoprotein A-I	7 (1)	519	30,759	APOA1_HUMAN APOA1_PONAB	0.73	0.033
4502	NI[b]					1.42	0.021
5903	NI					0.54	0.028
5905	Alpha-2-macroglobulin	1	81	163,188	A2MG_HUMAN	0.50	0.018
5908	NI					0.54	0.024
5910	Alpha-2-macroglobulin	2	129	163188	A2MG_HUMAN	0.52	0.018
161*	Alpha-1-antitrypsin	6	494	46,878	A1AT_HUMAN	0.66	0.028
	Vitronectin	4	271	55,069	VTNC_HUMAN		
	Angiotensinogen	3	193	53,438	ANGT_HUMAN		
369*	NI					0.41	0.004
445*	Ficolin-3	1	42	33,395	FCN3_HUMAN	0.50	0.044

Proteins identified by using Proteome Purify except those indicated with * that were analysed using ProteoMiner™.
[a] The intensity of each spot was normalized to the total intensity detected in the gel. Spots were matched between gels and the mean relative intensity of each spot in each group was calculated. The mean relative intensity of a spot in the IE group divided by the mean relative intensity of the spot in the non-IE group is listed as the fold change.
[b] NI = not identified.

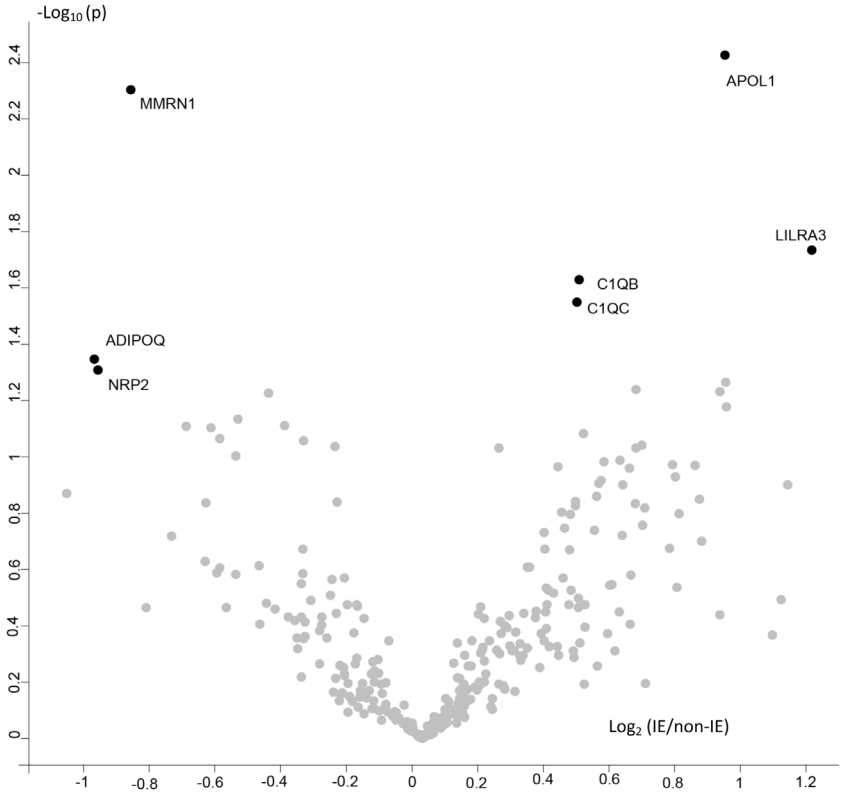

Figure 3. Vulcano plot of the 311 proteins detected in at least 70% of the samples in each group with the LFQ LC-MS/MS analysis based on the ProteoMiner™ Enrichment Kit. The x-axis shows the fold-change ($\log_2$ scale) of the IE group versus the non-IE group and the y-axis shows $-\log_{10}$ (p)-values from students t test. Seven proteins were significantly differentially regulated (indicated with black spots). Identifications are given in Table 3.

The combined proteomics analysis revealed a set of proteoforms/protein groups with significantly different levels in the IE versus the non-IE group. A STRING bioinformatic analysis (Szklarczyk et al., 2015) using the combined set of proteins observed to be perturbed from ELISA, 2D-PAGE and LFQ LC-MS/MS revealed that the proteins are biologically related to a significantly higher degree than expected (Figure 4). Ten of the proteins belong to the Biological Process 'Response to stress' and six belong to: 'Immune response'.

Table 3
Identification of differentially expressed protein groups in patients with IE vs. non-IE by LFQ LC-MS/MS using ProteoMiner™.

Identification	Peptides	Mr (kDa)	SwissProt	Fold change (IE/non-IE)[a]	*p*-Value
Apolipoprotein L1	9	44.0	APOL1_HUMAN	1.90	0.004
Complement C1q subcomponent subunit B	7	26.7	C1QB_HUMAN	1.39	0.024
Complement C1q subcomponent subunit C	7	25.8	C1QC_HUMAN	1.39	0.028
Leukocyte immunoglobulin-like receptor subfamily A member 3***	7	47.5	LIRA3_HUMAN	2.28	0.019
Neuropilin-2**	5	104.9	NRP2_HUMAN	0.50	0.049
Multimerin-1*	17	138.1	MMRN1_HUMAN	0.54	0.005
Adiponectin	6	26.4	ADIPO_HUMAN	0.50	0.045

The proteins were detected in 100% of samples, except when indicated, in at least 90% (*), 80% (**) and 70% (***) of all samples in each group.
[a] The fold-changes of the protein groups were calculated by taking the mean in the IE group divided by the mean in the non-IE group.

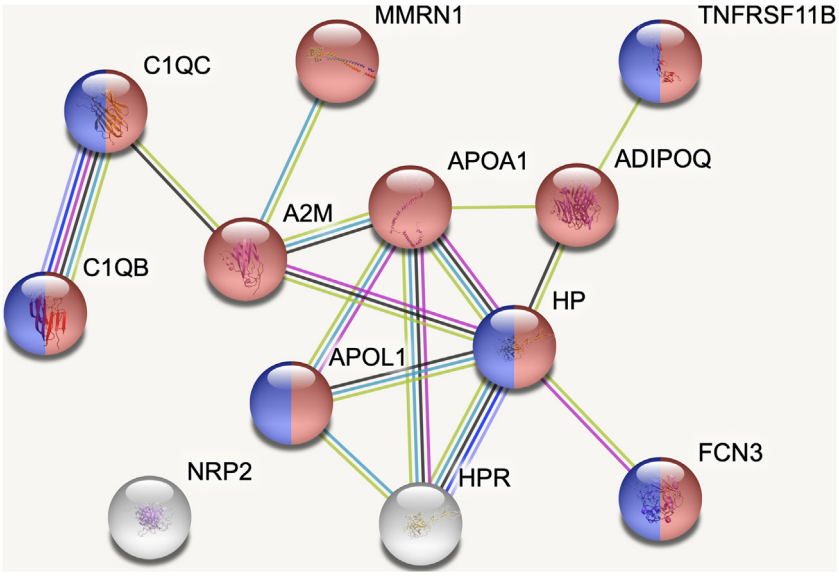

Figure 4. STRING analysis of the perturbed proteins found by proteomics. APOA1 (Apolipoprotein A–I), FCN3 (Ficolin-3), TNFRSF11B (OPG), C1QB (Complement C1q subcomponent subunit B), APOL1 (Apolipoprotein L1), A2M (Alpha-2-macroglobulin), HP (Haptoglobin), HPR (Haptoglobin-related proteins), NRP2 (Neuropilin-2), C1QC (Complement C1q subcomponent subunit C), MMRN1 (Multimerin-1), ADIPOQ (Adiponectin). There are 15 edges between the 12 proteins, which is higher than expected ($p < 1 \times 10^{-16}$). The proteins are biologically related to a significantly higher degree than expected for a random set of proteins. Ten of the perturbed proteins belong to the Biological Process 'Response to stress' (red) and six to 'Immune response' (blue).

Discussion

Patients and key findings

The aim of the present study was to identify putative biomarkers for diagnosis of IE in an unselected population of patients with suspected IE. The patients studied here were recruited for a previous study consisting of 759 patients (Knudsen et al., 2010), 147 patients with definite or possible IE where 80% had positive blood cultures and 612 patients excluded for having IE where 24% had positive blood cultures. From the previous group it was possible to perform ELISA measurements for fibulin-1 on 696 patents and for OPG on 689 patients. Furthermore, it was possible to perform proteomic analyses on 24 patients, 12 IE patients and 12 non-IE patients, all having bacteremia. In each group, 4 patients had Staphylococcus aureus, 2 patients had Enterococcus faecium and 6 patients had Streptococcus species. OPG levels were increased in IE patients as compared with non-IE patients; however fibulin-1 did not differentiate between groups, despite the fact that it is found in heart valves and previously was found to be associated with heart disease (Kruger et al., 2014). 2D-PAGE and LC-MS/MS analysis identified a set of proteoforms or protein groups as potential markers for IE.

Osteoprotegerin (OPG)

OPG is a glycoprotein produced in different tissues, including cardiovascular structures (Dhore et al., 2001; Nybo and Rasmussen, 2008; Schoppet et al., 2002). OPG is associated with atherosclerosis (Hofbauer and Schoppet, 2004) and risk factors of coronary artery disease (CAD) (Crisafulli et al., 2005; Hosbond et al., 2012; Jono et al., 2002; Mogelvang et al., 2012; Palazzuoli et al., 2008; Ren et al., 2008; Schoppet et al., 2003). In patients with aortic stenosis, OPG carries prognostic information, possibly because of its role in the calcification process (Dahl et al., 2013). Our OPG measurements were significantly higher in IE patients. These findings may suggest that OPG could be a biomarker to exclude IE in IE suspected patients. Selected combinations of sensitivity, specificity, positive predictive value and negative predictive value for OPG are given in the Supplementary Table 3. The optimal cut-off value has to be defined. In that context it is necessary to identify a cut-off value with either a high sensitivity or a high negative predictive value. Also, its use in a multi-marker strategy could be considered.

Proteomic analyses

We performed a number of proteomic analyses on 24 serum samples using different preparation techniques and different

analysis tools, i.e., 2D-PAGE and LFQ LC-MS/MS. 2D-PAGE is essentially a top-down procedure quantifying the intact proteins based on image analysis before identification while LFQ LC-MS/MS is a bottom-up procedure where the proteins for technical reasons are digested with trypsin before identification and quantification by MS signal intensities. Thus, due to technical differences the techniques are able to focus on different details. One of the obvious strengths with 2D-PAGE is that it is able to separate a post-translationally modified protein into several spots, even though the specific modifications are unknown. In general, plasma proteins migrate in 2D gels in several spots as a result of several types of post-translational modifications, especially glycosylations. Thus, a concentration change in one spot may reflect a concentration change in one specific proteoform (Smith and Kelleher, 2013) encoded from the same gene and not necessarily a large concentration change of the whole group of proteoforms encoded. The 2D-PAGE analysis performed here revealed concentration changes in a number of protein spots when comparing the IE group with the non-IE group. Thus, we found significant downregulation of proteoforms of haptoglobin, haptoglobin-related protein, α-2-macroglobulin, apolipoprotein A-I and ficolin-3 in the IE group. The LFQ LC-MS/MS method, on the other hand, does not distinguish between specific proteoforms of the proteins as we have performed it here but rather handles all proteoforms encoded from a gene as a protein group. With the LFQ LC-MS/MS technique we did not find significant changes in these proteins when analyzed as groups. However, with the latter technique we detected significant downregulation of the protein groups neuropilin-2, multimerin-1 and adiponectin while the protein groups apolipoprotein L1, complement C1q subcomponent subunits B and C and leukocyte immunoglobulin-like receptor subfamily A member 3 were upregulated.

α-2 macroglobulin is involved in the hemostatic process. Haptoglobin is known to bind hemoglobin. Ficolin-3 is known to be a recognition molecule of the lectin pathway and is reported to be associated with chronic heart failure severity and long-term outcome (Garred et al., 2009; Prohaszka et al., 2013). A large part of the proteins participate in the immune system. Further studies, however, are needed to clarify the specific implications in IE. A STRING bioinformatic analysis (Szklarczyk et al., 2015) using the combined set of 13 proteoforms/protein groups revealed enrichment of pathways related to stress and to the immune system.

Previous studies on IE and biomarkers have focused on procalcitonin (Knudsen et al., 2010; Mueller et al., 2004; Watkin et al., 2007; Yu et al., 2013), N-terminal pro-brain natriuretic peptide (NT-proBNP) (Bjurman et al., 2012; Kahveci et al., 2007; Shiue et al., 2010), cystatin C (Bjurman et al., 2012), troponins (Purcell et al., 2008; Watkin et al., 2004), lipopolysaccharide-binding protein (Vollmer et al., 2009; Watkin et al., 2007), S100A11 (Thuny et al., 2012b), aquaporin-9 (Thuny et al., 2012b) and E-selectin (Soderquist et al., 1999). However, no studies have previously investigated IE patients with an appropriate, comparable control group, i.e., patients with bacteremia and excluded IE (Snipsøyr et al., 2016) as in the present study. Secondly, OPG and fibulin-1 have not previously been evaluated. A thrombotic tendency in IE patients has been reported (Fukuda et al., 1982). One of the findings in that study was decreased plasma level of antithrombin III in IE-patients, contributing to a hyper coagulable tendency. However, with our proteomic analysis using direct LC-MS/MS, we could not detect a massive significant decrease in the level of the protein group antithrombin III in the IE group (results not shown). We found perturbations in the levels of other proteins, i.e., a proteoform of α-2-macroglobulin and the protein group multimerin-1 that might affect the coagulation process.

Study limitations

Our hypothesis was to find biological markers originating from damaged heart valves due to IE. However due to the small size of a heart valve the concentration in the blood stream may be very low and thereby difficult to detect. This could be circumvented by performing analyses directly on the heart valves to detect changes in proteins that subsequently might be detected in the blood stream. Also, there are technical limitations to the study. The 2D-PAGE technique may identify changes in a few proteoforms with specific modifications while the LFQ LC-MS/MS analysis measures groups of several proteoforms. The necessary efforts to deplete samples for high abundant proteins may partly remove some lower abundant attached proteins and the step that enriches for lower abundant proteins (ProteoMiner™) also levels out the concentration differences, thereby diminishing the observed differences. Each of the techniques are capable of identifying only a subset of all the proteins present, e.g., OPG was not identified by the direct LC-MS/MS technique.

Conclusions

At present, no biomarker is being used in the diagnosis of IE, and this is partly due to low specificity, but also some of the studies require verification of results in larger cohorts before they can be included in everyday clinical practice (Snipsøyr et al., 2016). Finally, we want to stress that the markers given here are tentative only and their utility in the clinical setting should be verified with independent studies. The combined proteomic analyses performed have detected a number of proteins that change in patients with IE, ten that respond to stress and six belonging to the immune response. Although these findings need to be verified, our observations may indicate an impact on these systems. Further studies are necessary to establish their specific roles. The concentration of OPG was increased in IE patients. Its suitability as a marker used alone is restricted due to low sensitivity and specificity. However, its suitability used in combination with other putative markers in a multi-marker strategy remains to be clarified.

Scientific ethical approval

This study is an extension of a previously described study (Knudsen et al., 2010) approved by the Scientific Ethical Committee Central Region Denmark, the Danish Data Protection Agency (j. nr. 2013-41-2139) and was reported to ClinicalTrials.gov (identifier NCT00524212). The present analyses of blood samples from the biobank was reapproved by the Scientific Ethical Committee Central Region Denmark. All experiments were performed in accordance with relevant guidelines and regulations. Written informed consent was obtained from all participating patients.

Author contributions

M.G.S, H.W., E.P., B.H. designed the study. M.G.S., H.W., A.S., S.H.P., L.M.R., E.P., B.H. acquired data. M.G.S., H.W., M.L., A.S., L.M.R., E.P., B.H. analyzed data. M.G.S., H.W., M.L., H.V., S.H.P., L.M.R., E.P., B.H. interpreted data. M.G.S., H.W., E.P., B.H. drafted the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Acknowledgements

The authors would like to thank Inge Kjærgaard Nielsen, Mona Britt Hansen, Lone Pødenphant and Anne-Marie Jakobsen for expert technical assistance. This work was supported by the Danish Heart Foundation (MGS), the John and Birthe Meyer Foundation (BH), the Eiler and Tatiana Filtenborg scholarship (MGS), the Copenhagen University fund for medical students (MGS) and the MEMBRANE Center at Aarhus University (BH). The Obelske family foundation, the Svend Andersen Foundation and the Spar Nord foundation are acknowledged for grants to the analytical platform enabling parts of this study (AS). The Danish Agency for Science and Higher Education (AS) is acknowledged for the funding to Danish National Mass Spectrometry Platform for Functional Proteomics enabling parts of this study (PRO-MS; grant no. 5072-00007B). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al., 2016) partner repository with the dataset identifier PXD010149.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijid.2020.02.026.

References

Argraves WS, Tanaka A, Smith EP, Twal WO, Argraves KM, Fan D, et al. Fibulin-1 and fibrinogen in human atherosclerotic lesions. Histochem Cell Biol 2009;132 (5):559–65.
Beynon RP, Bahl VK, Prendergast BD. Infective endocarditis. BMJ (Clin Res ed) 2006;333(7563):334–9.
Bjurman C, Snygg-Martin U, Olaison L, Fu ML, Hammarsten O. Cystatin C in a composite risk score for mortality in patients with infective endocarditis: a cohort study. BMJ Open 2012;2(4).
Bouza E, Menasalvas A, Munoz P, Vasallo FJ, del Mar Moreno M, Garcia Fernandez MA. Infective endocarditis—a prospective study at the end of the twentieth century: new predisposing conditions, new etiologic agents, and still a high mortality. Medicine 2001;80(5):298–307.
Cangemi C, Skov V, Poulsen MK, Funder J, Twal WO, Gall MA, et al. Fibulin-1 is a marker for arterial extracellular matrix alterations in type 2 diabetes. Clin Chem 2011;57(11):1556–65.
Crisafulli A, Micari A, Altavilla D, Saporito F, Sardella A, Passaniti M, et al. Serum levels of osteoprotegerin and RANKL in patients with ST elevation acute myocardial infarction. Clin Sci (Lond, Engl: 1979) 2005;109(4):389–95.
Dahl JS, Videbaek L, Poulsen MK, Rudbaek TR, Christensen NL, Pellikka PA, et al. Relation of osteoprotegerin in severe aortic valve stenosis to postoperative outcome and left ventricular function. Am J Cardiol 2013;112(9):1433–8.
Delahaye F, Goulet V, Lacassin F, Ecochard R, Selton-Suty C, Hoen B, et al. Characteristics of infective endocarditis in France in 1991. A 1-year survey. Eur Heart J 1995;16(3):394–401.
Dhore CR, Cleutjens JP, Lutgens E, Cleutjens KB, Geusens PP, Kitslaar PJ, et al. Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. Arterioscler Thromb Vasc Biol 2001;21(12):1998–2003.
Durack DT, Lukes AS, Bright DK. New criteria for diagnosis of infective endocarditis: utilization of specific echocardiographic findings. Duke Endocarditis Service. Am J Med 1994;96(3):200–9.
Farrah T, Deutsch EW, Omenn GS, Campbell DS, Sun Z, Bletz JA, et al. A high-confidence human plasma proteome reference set with estimated concentrations in PeptideAtlas. Mol Cell Proteomics 2011;10(9) M110 006353.
Fukuda Y, Kuroiwa Y, Tabuchi H, Ohshige T, Sanada J, Minami Y, et al. A thrombotic tendency in patients with infective endocarditis. Jpn Circ J 1982;46(5):460–7.
Garred P, Honore C, Ma YJ, Munthe-Fog L, Hummelshoj T. MBL2, FCN1, FCN2 and FCN3-The genes behind the initiation of the lectin pathway of complement. Mol Immunol 2009;46(14):2737–44.
Griffin MR, Wilson WR, Edwards WD, O'Fallon WM, Kurland LT. Infective endocarditis. Olmsted County, Minnesota, 1950 through 1981. JAMA 1985;254(9):1199–202.
Hansen ML, Dahl JS, Argraves WS, Irmukhamedov A, Rasmussen LM. Aortic valve stenosis and atrial fibrillation influence plasma fibulin-1 levels in patients treated with coronary bypass surgery. Cardiology 2013;126(3):202–6.
Hofbauer LC, Schoppet M. Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular diseases. JAMA 2004;292(4):490–5.
Hosbond SE, Poulsen TS, Diederichsen AC, Nybo M, Rasmussen LM, Mickley H. Osteoprotegerin as a marker of atherosclerosis: a systematic update. Scand Cardiovasc J 2012;46(4):203–11.
Issa VS, Fabri Jr. J, Pomerantzeff PM, Grinberg M, Pereira-Barreto AC, Mansur AJ. Duration of symptoms in patients with infective endocarditis. Int J Cardiol 2003;89(1):63–70.
Jono S, Ikari Y, Shioi A, Mori K, Miki T, Hara K, et al. Serum osteoprotegerin levels are associated with the presence and severity of coronary artery disease. Circulation 2002;106(10):1192–4.
Kahveci G, Bayrak F, Mutlu B, Bitigen A, Karaahmet T, Sonmez K, et al. Prognostic value of N-terminal pro-B-type natriuretic peptide in patients with active infective endocarditis. Am J Cardiol 2007;99(10):1429–33.
Kamper P, Ludvigsen M, Bendix K, Hamilton-Dutoit S, Rabinovich GA, Moller MB, et al. Proteomic analysis identifies galectin-1 as a predictive biomarker for relapsed/refractory disease in classical Hodgkin lymphoma. Blood 2011;117 (24):6638–49.
Knudsen JB, Fuursted K, Petersen E, Wierup P, Molgaard H, Poulsen SH, et al. Infective endocarditis: a continuous challenge. The recent experience of a European tertiary center. J Heart Valve Dis 2009;18(4):386–94.
Knudsen JB, Fuursted K, Petersen E, Wierup P, Molgaard H, Poulsen SH, et al. Procalcitonin in 759 patients clinically suspected of infective endocarditis. Am J Med 2010;123(12):1121–7.
Kruger R, Rasmussen LM, Argraves WS, Eugen-Olsen J, Nielsen OW, Blyme A, et al. Extracellular matrix biomarker, fibulin-1, is closely related to NT-proBNP and soluble urokinase plasminogen activator receptor in patients with aortic valve stenosis (the SEAS study). PLOS ONE 2014;9(7):e101522.
Li JS, Sexton DJ, Mick N, Nettles R, Fowler Jr. VG, Ryan T, et al. Proposed modifications to the Duke criteria for the diagnosis of infective endocarditis. Clin Infect Dis 2000;30(4):633–8.
Mogelvang R, Pedersen SH, Flyvbjerg A, Bjerre M, Iversen AZ, Galatius S, et al. Comparison of osteoprotegerin to traditional atherosclerotic risk factors and high-sensitivity C-reactive protein for diagnosis of atherosclerosis. Am J Cardiol 2012;109(4):515–20.
Mueller C, Huber P, Laifer G, Mueller B, Perruchoud AP. Procalcitonin and the early diagnosis of infective endocarditis. Circulation 2004;109(14):1707–10.
Mylonakis E, Calderwood SB. Infective endocarditis in adults. N Engl J Med 2001;345 (18):1318–30.
Nybo M, Rasmussen LM. The capability of plasma osteoprotegerin as a predictor of cardiovascular disease: a systematic literature review. Eur J Endocrinol/Eur Fed Endocr Soc 2008;159(5):603–8.
Palazzuoli A, Rizzello V, Calabro A, Gallotta M, Martini G, Quatrini I, et al. Osteoprotegerin and B-type natriuretic peptide in non-ST elevation acute coronary syndromes: relation to coronary artery narrowing and plaques number. Clin Chim Acta: Int J Clin Chem 2008;391(1–2):74–9.
Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 1999;20(18):3551–67.
Prohaszka Z, Munthe-Fog L, Ueland T, Gombos T, Yndestad A, Forhecz Z, et al. Association of ficolin-3 with severity and outcome of chronic heart failure. PLOS ONE 2013;8(4):e60976.
Purcell JB, Patel M, Khera A, de Lemos JA, Forbess LW, Baker S, et al. Relation of troponin elevation to outcome in patients with infective endocarditis. Am J Cardiol 2008;101(10):1479–81.
Ren MY, Sui SJ, Zhang Y, Xu FY, Xu XQ, Zhao JJ, et al. Increased plasma osteoprotegerin levels are associated with the presence and severity of acute coronary syndrome. Acta Cardiol 2008;63(5):615–22.
Schoppet M, Preissner KT, Hofbauer LC. RANK ligand and osteoprotegerin: paracrine regulators of bone metabolism and vascular function. Arteriosc Thromb Vasc Biol 2002;22(4):549–53.
Schoppet M, Sattler AM, Schaefer JR, Herzum M, Maisch B, Hofbauer LC. Increased osteoprotegerin serum levels in men with coronary artery disease. J Clin Endocrinol Metab 2003;88(3):1024–8.
Shiue AB, Stancoven AB, Purcell JB, Pinkston K, Wang A, Khera A, et al. Relation of level of B-type natriuretic peptide with outcomes in patients with infective endocarditis. Am J Cardiol 2010;106(7):1011–5.
Smith LM, Kelleher NL. Consortium for Top Down Proteomics. Proteoform: a single term describing protein complexity. Nat Methods 2013;10(3):186–7.
Snipsøyr MG, Ludvigsen M, Petersen E, Wiggers H, Honoré B. A systematic review of biomarkers in the diagnosis of infective endocarditis. Int J Cardiol 2016;202:564–70.
Soderquist B, Sundqvist KG, Vikerfors T. Adhesion molecules (E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)) in sera from patients with Staphylococcus aureus bacteraemia with or without endocarditis. Clin Exp Immunol 1999;118 (3):408–11.
Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, et al. STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucl Acids Res 2015;43(Database issue):D447–52.
Thuny F, Grisoli D, Collart F, Habib G, Raoult D. Management of infective endocarditis: challenges and perspectives. Lancet 2012a;379(9819):965–75.
Thuny F, Textoris J, Amara AB, Filali AE, Capo C, Habib G, et al. The gene expression analysis of blood reveals S100A11 and AQP9 as potential biomarkers of infective endocarditis. PLoS ONE 2012b;7(2):e31490.
Tyanova S, Temu T, Cox J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. Nat Protoc 2016a;11(12):2301–19.
Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, et al. The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat Methods 2016b;13(9):731–40.

Ueland T, Aukrust P, Dahl CP, Husebye T, Solberg OG, Tonnessen T, et al. Osteoprotegerin levels predict mortality in patients with symptomatic aortic stenosis. J Intern Med 2011;270(5):452–60.
Vizcaino JA, Csordas A, del-Toro N, Dianes JA, Griss J, Lavidas I, et al. 2016 update of the PRIDE database and its related tools. Nucl Acids Res 2016;44 (D1):D447–56.
Vollmer T, Piper C, Kleesiek K, Dreier J. Lipopolysaccharide-binding protein: a new biomarker for infectious endocarditis?. Clin Chem 2009;55 (2):295–304.
Watkin RW, Harper LV, Vernallis AB, Lang S, Lambert PA, Ranasinghe AM, et al. Pro-inflammatory cytokines IL6, TNF-alpha, IL1beta, procalcitonin, lipopolysaccharide binding protein and C-reactive protein in infective endocarditis. J Infect 2007;55 (3):220–5.
Watkin RW, Lang S, Smith JM, Elliott TS, Littler WA. Role of troponin I in active infective endocarditis. Am J Cardiol 2004;94(9):1198–9.
Yu CW, Juan LI, Hsu SC, Chen CK, Wu CW, Lee CC, et al. Role of procalcitonin in the diagnosis of infective endocarditis: a meta-analysis. Am J Emerg Med 2013;31 (6):935–41.