



Exercise-Based Cardiac Rehabilitation Reduces Key Inflammatory Biomarkers in Atherosclerosis: A Dose Response Study

Malene Munk Jørgensen^{1,2*}, Annemette Krintel Petersen^{2,3}, Mette Mølby Nielsen¹, Mikkel Steen Petersen⁴, Bjarne Kuno Møller⁴, Kristine Møller² and Sussie Laustsen^{3,5}

¹VIA Faculty of Health Science, VIA University College, Denmark

²Department of Physiotherapy and Occupational therapy, Aarhus University Hospital, Denmark

³Department of Clinical Medicine, Aarhus University, Denmark

⁴Department of Clinical Immunology, Aarhus University Hospital, Denmark

⁵Department of Cardiothoracic and Vascular Surgery, Aarhus University Hospital, Denmark

Abstract

Supervised Exercise-based Cardiac Rehabilitation (ECR) is recommended for patients with Ischemic Heart Disease (IHD). Vascular inflammation is an important pathophysiological factor in development of IHD and cardiovascular events. The aim of this study was to investigate whether an extended 12-week ECR program was more successful in reducing low-grade vascular inflammation, compared to a conventional 8-week ECR program. A total of 110 patients treated for IHD, and referred to ECR, were randomized to a 12-week or 8-week ECR intervention. We measured the concentration of soluble vascular cell adhesion molecule 1 (sVCAM-1), interleukin-6 (IL-6), high-sensitivity C-reactive protein (hsCRP), before and after participation in either of the two ECRs and at 6-month and one year follow-up. Additionally, we determined the absolute White Blood Cell count (WBC), the absolute monocyte count, and the monocyte sub populations. For the primary outcome (sVCAM-1), we observed no differences between groups on a short and long-term basis. At 1-year follow-up, there were no differences in IL-6, hsCRP, WBC and monocytes. However, in the 12-week ECR group more inflammatory biomarkers were significantly reduced at the end of ECR (IL-6: $p=0.002$; hsCRP: $p<0.00001$; CD14⁺⁺CD16⁻ classical monocytes: $p=0.0178$), compared to the 8-week ECR group. On a short time basis, 12-week ECR reduces significantly more inflammatory biomarkers in comparison with an 8-week ECR program. However, this effect was not sustained on a long-term basis, probably due to an underpowered study design. Further studies on optimizing ECR program duration and frequency are needed to evaluate the effects for the vascular inflammatory biomarkers.

Keywords: Atherosclerosis; Exercise-based cardiac rehabilitation; Inflammation; IL-6; sVCAM-1; hsCRP

Introduction

Exercise-based Cardiac Rehabilitation (ECR) in patients with Ischemic Heart Disease (IHD) is effective and safe. Moreover, ECR reduces cardiovascular morbidity and mortality, underlining the importance of establishing and improving effective ECR programs [1], which already play an important role in Cardiac Rehabilitation (CR) [2]. The importance for patients with cardiac disease to participate in a supervised ECR program is well-known [3]; however, evidence is insufficient regarding training frequency and duration of ECR programs in patients with IHD [1,4]. ECR reduces the risk of cardiovascular events and suppresses vascular inflammation. Inflammation is an important pathophysiological factor in the development of IHD and cardiovascular events [5]. It is known that physical exercise reduces vascular inflammation in patients with IHD [6]. Vascular cell adhesion molecule-1 (VCAM-1) is expressed during pro-atherosclerotic conditions and plays a critical role in atherosclerosis. The soluble form of VCAM-1 (sVCAM-1) is a known biomarker of endothelial dysfunction. Seemingly, there is moderate evidence that aerobic physical exercise reduces the sVCAM-1 concentration, and that this reduction indicates reduced vascular inflammation [7,8]. Interleukin 6 (IL-6) is considered an upstream inflammatory cytokine playing

OPEN ACCESS

*Correspondence:

Malene Munk Jørgensen, VIA Faculty of Health Science, VIA University College, Denmark,

E-mail: mmj@via.dk

Received Date: 16 Mar 2018

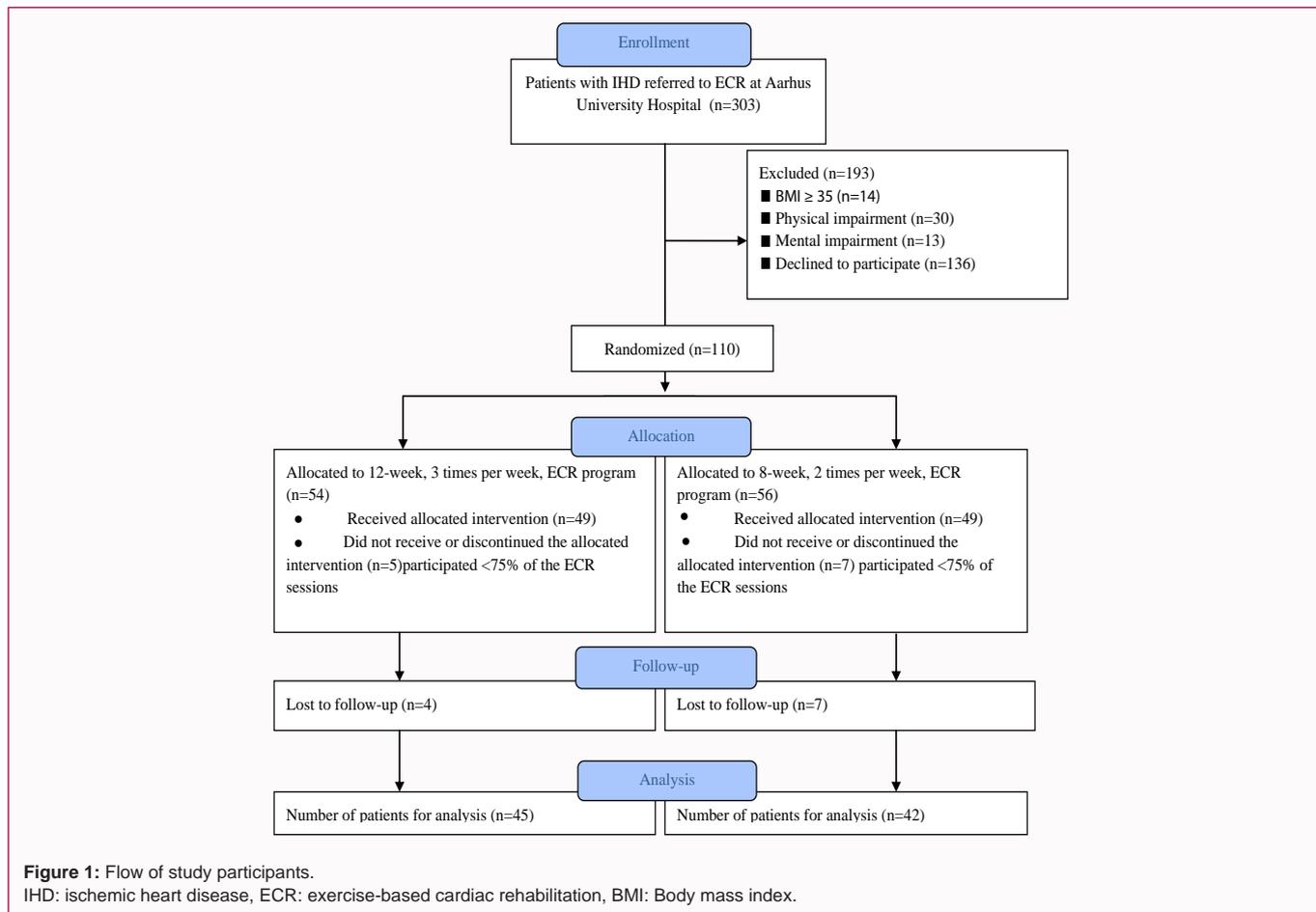
Accepted Date: 16 Apr 2018

Published Date: 23 Apr 2018

Citation:

Jørgensen MM, Petersen AK, Nielsen MM, Petersen MS, Møller BK, Møller K, et al. Exercise-Based Cardiac Rehabilitation Reduces Key Inflammatory Biomarkers in Atherosclerosis: A Dose Response Study. *Ann Atheroscler Res.* 2018; 1(3): 1012.

Copyright © 2018 Malene Munk Jørgensen. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.



a central role in propagating the downstream inflammatory response accountable for atherosclerosis. High levels of circulating IL-6 induce a number of effects, including hepatic CRP synthesis, promotion of monocyte differentiation, activation of endothelial cells, and increased coagulation [9]. Thus, circulating IL-6 is established as a biomarker for risk stratification in patients with unstable IHD [10,11]. Fluctuations in inflammatory biomarkers such as high-sensitivity C-Reactive Protein (hsCRP), predict enhanced cardiovascular risk [12]. In patients with stable IHD an increased level of hsCRP is independently associated with an increased risk of heart failure [13] and death [14]. The development of a high-sensitivity assay has provided a biomarker-based tool for risk assessment, and guidelines from professional medical societies now incorporate hsCRP into risk prediction algorithms for cardiovascular events [15,16]. Absolute White Blood Cell count (WBC) is an independent risk factor and predictor of future cardiovascular events [17]. Moreover, high monocyte counts also correlate with peripheral endothelial dysfunction. Monocytes are involved in the initiation and progression of atherosclerosis, especially when transforming into lipid-loaded macrophages. The involvement of monocytes in atherosclerosis is complex and includes several processes. Monocytes have been shown to play a role during the long-term process of initiation and formation of atherosclerotic plaque during the acute inflammatory process following Acute Coronary Syndrome (ACS), and during post ACS recovery [18]. Human monocytes constitute a heterogeneous population according to gene expression and function, and several distinct features of the monocyte are important in explaining this multitude of actions. At least three human monocyte subsets have been identified: Classical

Table 1: Baseline characteristics of the study population.

	8-week ECR n=54	12-week ECR n=56	p-value
Gender, n (%)			
Female	10 (18.5)	11 (19.6)	0.9 ¹
Male	44 (81.5)	45 (80.4)	
Age (years) mean/SD	62.2 (9.8)	62.7 (8.4)	0.8 ²
BMI (kg/m ²)	28 (3.8)	28 (3.6)	0.7 ²
Current smoker, n (%)			
Yes	5 (9%)	1 (2%)	0.08 ¹
No	49 (91%)	55 (98%)	
Diabetes mellitus, n (%)	10 (18.5%)	11 (19.6%)	0.9 ¹
Hypertension, n (%)	36 (67%)	37 (68.5%)	0.9 ¹
Hypercholesterolemia, n (%)	28 (52%)	36 (64%)	0.2 ¹
Chronic kidney disease, n (%)	4 (7.4%)	2 (3.6%)	0.4 ¹
Statins, n (%)	49 (91%)	49(88%)	0.5 ¹
Polypharmacy, n (%)	26 (48%)	32 (57%)	0.18 ¹
Aerobic capacity (watts) mean/SD	134 (46)	140 (45)	0.5 ²

¹χ²-test, ² t-test BMI: Body mass index, SD: Standard deviation

monocytes (CD14⁺⁺CD16⁻), intermediate monocytes (CD14⁺⁺CD16⁺), and non-classical monocytes (CD14⁺CD16⁺⁺) [19]. Approximately 90% of the circulating monocytes are classical monocytes. The populations of CD16⁺ monocytes, including the CD14⁺⁺CD16⁺ and the CD14⁺CD16⁺⁺ subtypes, are considered pro-inflammatory. They exhibit an increased production of inflammatory cytokines elevated in chronic inflammatory diseases [20,21]. Today, the intermediate monocyte subset is considered a separate and unique subset [22,23].

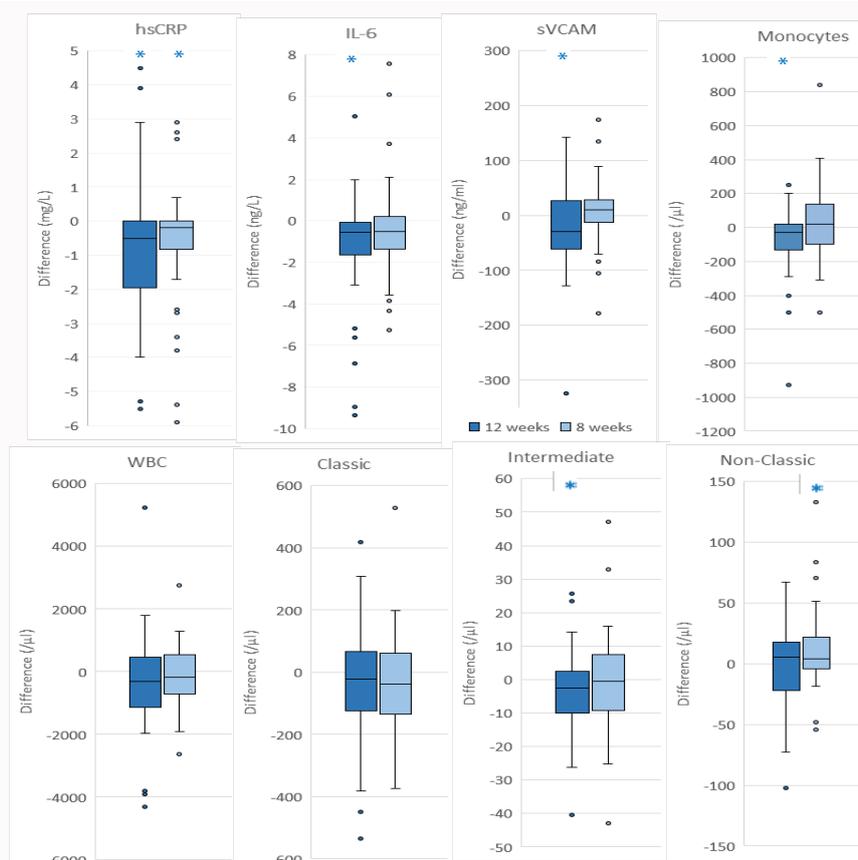


Figure 2: Differences in biomarker concentrations and leucocyte counts within groups, before and after completed intervention. Solid line indicates median difference. The box indicates the 25th and 75th percentiles and the error bars the 10th and 90th percentiles. Significant changes are marked by *. hsCRP (High sensitivity C-reactive protein), IL-6 (Interleukin 6), sVCAM (Soluble vascular adhesion molecule-1), Monocyte (Total monocyte count), WBC (Absolute Leukocyte count), Classic (Classical monocyte subtype CD14⁺⁺CD16⁻), Intermediate (Intermediate monocytes (CD14⁺⁺CD16⁺), and Non-classic (Non-classical monocyte subtype CD14⁺CD16⁺⁺).

The role of the individual subsets in cardiovascular disease is not fully understood, but has recently been shown to play a specific role in the pathogenesis of atherosclerosis and ability to independently predict cardiovascular events [24,25]. However, a recent study proposed that all subsets may be important, and an entire shift in the monocyte profile is more informative than the final count of the three subtypes [26,27]. In summary, a number of key inflammatory markers seem to predict the prognosis of atherosclerosis in patients with IHD. Unfortunately, the change of inflammatory biomarkers during ERC programs has not been determined. The aim of this study was to investigate whether an extended 12-week ECR program was more successful in reducing low-grade inflammation compared with a conventional 8-week ECR program among patients with IHD.

Materials and Methods

Subjects

A total of 303 patients diagnosed with IHD were recruited among patients referred to outpatient ECR at the Department of Physiotherapy and Occupational Therapy, Aarhus University Hospital, Denmark between March 2011 and October 2012. Inclusion criteria were treatment with Coronary Artery Bypass Grafting (CABG) or Percutaneous Coronary Intervention (PCI). Exclusion criteria were BMI >35, and physical or mental impairment estimated to cause inability to fulfill the ECR program (Figure 1). The study was performed as a single-blinded, randomized, clinical trial with one

year follow-up. The included patients with IHD were randomized to either a conventional 8-week ECR program consisting of aerobic and muscle strength training for one hour twice a week or an extended 12-week ECR program consisting of aerobic and muscle strength training for one hour three times a week. A detailed description of the ECR programs can be found in the Trial Protocol under Supporting Information (www.clinicaltrials.gov). The ECR program was initiated 6-8 weeks after CABG and 2-4 weeks after PCI. Follow-up samples were obtained at six and twelve months after completion of the ECR program, in the period between December 2012 and January 2014. Baseline characteristics of the study population including gender, age, diagnoses, BMI, smoking, diabetes, history of acute myocardial infarction, and medication were collected from medical records and interviews. This study was the secondary analysis of a main RCT study investigating the effects of an optimized ECR program after treatment for cardiac disease. The main study showed that maximum oxygen uptake (VO_{2peak}) was significantly higher in the optimized group on a short and long time basis, compared to the conventional ECR group. (Submitted 2018-03-14: European Journal of Preventive Cardiology, Title: Effect of an extended exercise-based cardiac rehabilitation program; A Randomised Controlled Trial) Calculations related to the statistical power were done according to the expected differences between the two ERC programs in VO_{2peak} . The primary outcome measure in the present study was differences in sVCAM concentrations. Secondary outcomes were IL-6, hsCRP,

Table 2: Data on primary and secondary outcome measures in the 8-week group and the 12-week group, respectively at all measurement times (baseline, end of ECR, 6- and 12 months after end of the exercise-based cardiac rehabilitation program). Data are described by median and 95% confidence interval (CI). Differences between groups are presented as relative differences (%) with 95% CI.

	Baseline		End Exercise-based CR		6 months follow-up		12 months follow-up	
	Median [95% CI]	Relative difference (%)	Median [95% CI]	Relative difference (%)	Median [95% CI]	Relative difference (%)	Median [95% CI]	Relative difference (%)
Primary outcome								
sVCAM (ng/L)								
12-week n=42	673.3 [622.9;723.8]	8.7 [-17.6; 2.7] p = 0.137	650.7 [603.8;697.7]	3.7 [-13.3; 7.3] p=0.502	634.8 [603.8;697.7]	2.0 [-11.9; 9.1] p = 0.716	661.9 [614.4; 709.3]	5.0 [-14.3; 6] p = 0.368
8-week n=41	619.4 [572.3;666.6]		627.6 [581.3;673.8]		622.4 [576.9;667.9]		630.6 [584.5; 676.7]	
Secondary outcomes								
IL-6 (ng/L)								
12-week n=47	4.1 [3.2; 4.9]	3.9 [-29.8; 32.1] p = 0.483	2.8 [3.2; 4.9]	3.7 [-11.0; 57.0] p = 0.277	2.7 [2.3; 3.3]	29.1 [4; 91.4] p = 0.025	3.0 [2.4; 3.6]	14.5 [-11.6; 54.7] p = 0.259
8-week n=47	3.9 [3.0; 4.8]		3.9 [3.0; 4.8]		3.9 [3.0; 4.8]		3.5 [2.8; 4.2]	
IL-6 (ng/L) ^a								
12-week n= 44	3.4 [2.9; 3.9]	2.2 [-22.3; 23.2] p = 0.852	2.5 [2.2; 2.8]	9.3 [-9.8; 34.8] p = 0.338	2.4 [2.0; 2.9]	25.0 [3.1; 72.5] p = 0.028	2.8 [2.4; 3.2]	11.5 [-8.5; 39.7] p = 0.255
8-week n= 39	3.3 [2.8; 3.9]		2.8 [2.4; 3.2]		3.2 [2.6; 3.8]		3.1 [2.6; 3.6]	
hsCRP (mg/L)								
12-week n=47	2.7 [1.87;3.5]	3.9 [-39.1; 47.3] p = 0.809	1.5 [1.09;1.9]	8.6 [-26.7; 63.2] p= 0.659 1.2 [0.8; 1.5]	39.9 [10; 151.9] p = 0.016	1.6 [1.2; 2.1]	0.7 [-34.1; 54] p = 0.973	
8-week n=48	2.5 [1.7; 3.3]		1.6 [1.2; 2.1]		1.9 [1.4; 2.5]		1.6 [1.2; 2.1]	
hsCRP (mg/L) ^b								
12-week n=45	2.12.1 [1.6; 2.6]	13.7 [-38.6; 26.0] p = 0.480	1.3 [1.1; 1.6]	22.2 [-34.1; 25.2] p = 0.555	1.1 [0.8; 1.3]	32.8 [6; 109] p = 0.022	1.4 [1.1; 1.7]	0.04 [-29.1; 42.2] p = 0.998
8-week n= 37	1.8 [1.4; 2.3]		1.2 [0.9; 1.5]		1.6 (1.2; 2.0)		1.4 [1.0; 1.7]	
WBC (μL)								
12-week n=49	7057.9 [6548.5;7567.2]	13.4 [2; 20] p = 0.020	6726.9 [6236.6;7217.3]	9.8 [1.2;18.1] p = 0.083	6488.7 [6009.1; 6968.3]	7.2 [3.8; 16.2] p = 0.199	6608.7 [6101.9; 7115.5]	10.3 [1.27; 18.9] p = 0.082
8-week n=49	6224.4 [5755.7; 6693.1]		6124.5 [5658.7; 6590.4]		6050.29 [5587.3;6513.3]		5990.6 [5517.4;6463.9]	
Monocytes (1/μL) Sysmex								
12-week n=49	654.8 [586.3;723.2]	10 [-21.9; 5.9] p= 0.220	595.5 [540.2;650.8]	-6.1 [-6.9;22] p=0.356	613.9 [558.4;669.4]	4.2 [-8.5; 19] p =0.521	631.5 [574.8; 688.1]	4.0 [-8.5; 18.6] p = 0.521
8-week n=49	595.5 [530.5;660.4]		634.4 [573; 695.9]		640.7 [580.7; 700.6]		657.9 [597; 718.7]	
CD14 ⁺ CD16 ⁻ (/μL) (classical monocytes)								
12-week n=49	502.9 [443.26;562.5]	0.8 [-15.2; 19.8] p = 0.926	490.3 [444.6; 536.1]	3.2 [-15.5; 11] p=0.647	436.6 [388.4; 484.9]	15.9 [1.3; 39.5] p=0.035	432.6 [378.1; 487.1]	6.9 [-10.3; 28.7] p = 0.437
8-week n=49	507 [444.3; 569.7]		475.1 [428.8; 521.3]		518.9 [459.4; 578.4]		464.7 [404.8; 524.6]	
CD14 ⁺ CD16 ⁺ (/μL) (intermediate monocytes)								
12-week n=49	23.6 [19.8; 27.5]	21.4 [-4.4; 34.9] p = 0.107	20.6 [17.5; 23.6]	5.5 [-25.7; 14.8] p = 0.470	18.9 [15.9; 22]	19 [-2.1; 55.8] p=0.075	20.4 [17.1; 23.7]	1.2 [-19.9; 27.9] p = 0.922
8-week n=49	19.5 [16.2; 22.8]		19.5 [16; 21.9]		23.4 [19.5; 27.3]		20.6 [17.2; 24.1]	
CD14 ⁺ CD16 ⁺⁺ (/μL) (non classical monocytes)								
12-week n=49	53.7 [45.1; 62.4]	18.1 [-33.1;7.1] p = 0.164	51.1 [41.1; 61.1]	-3.8 [-25.7; 38.2] p = 0.788	49.7 [41.2; 58.2]	0.9 [-22.5; 26.7] p = 0.942	44.2 [36.7; 51.7]	0.9 [-22.4;26.7] p = 0.945
8-week n=49	45.5 [37.8; 53.1]		53.1 [42.2; 64]		49.2 [40.6; 57.9]		43.8 [36.2; 51.4]	

CR: Cardiac rehabilitation

^aconcentrations above 10 pg/mL removed

^bconcentrations above 8 mg/ml removed

WBC, monocytes and classical -, intermediate -, and non-classical monocytes. All primary and secondary outcome measures were sampled at start of ECR, at the end of ECR, and at six months and one year after completion of the ECR program. The study was approved by The Danish Data Protection Agency (1-16-02-6-11) and the Central Denmark Regional Committees on Health Research Ethics (28 098) and complies with the declaration of Helsinki. The trial has been registered at ClinicalTrials.gov (NCT01617850). Oral and written informed consent was obtained from each participant.

Immunoassays

Before entering the ECR programs, venous blood was collected in 4-m Lithium-heparin tubes from resting non-fasting participants. The samples were incubated at 4°C, stored for a maximum of two hours before plasma isolation, and preserved at -80°C until analysis. All the samples were analyzed simultaneously at the end of the follow-up period. sVCAM-1 concentrations were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) from R&D Systems (Quantakine® ELISA, Human sVCAM-1/CD106, DVC00). IL-6 concentrations were measured using automated electrochemiluminescence assay (IL-6, 05109442, Roche) and the Cobas 6000® analyzer, e-module (Roche Diagnostics). HsCRP concentrations were measured using an automated immunoassay (hsCRP, Roche) and the Cobas 6000 c-module. Limits of quantitation were: IL-6: 1.5pg/mL, hsCRP: 0.6 mg/L. Samples were analyzed in duplicates according to manufacturer's instructions and inter-assay variation was 2.2%. All concentration determinations were performed blinded to the ECR programs. hsCRP concentrations above 8 mg/ml, IL-6 concentrations above 10pg/mL and sVCAM concentrations above 1.2 µg/ml were classified as outliers to avoid including patients suffering from undiagnosed or unreported inflammatory conditions.

Flow cytometry

Venous blood was collected in 2.7 mL sodium-citrate tubes. All samples were assigned a sample number and all analyses and data collection were performed blinded to the ECR programs. A complete blood count including an automated cell differentiation was performed with an automated hematology analyzer (Sysmex XE-2100, Sysmex).

Monocyte sub typing was performed in a whole-blood lysis-no-wash assay using 50µL sodium citrate anti coagulated blood. Cells were stained with anti-CD14 [APC-conjugated] (clone RM052, Beckman Coulter), anti-CD16-PE (clone 3G8, Beckman Coulter), and anti-CD62L [FITC] (clone DREG56, Beckman Coulter) (although all samples were stained with anti-CD62L and compensated accordingly, the CD62L-staining proved unnecessary and was not used in the final gating strategy). A total of 50 µL blood was incubated with relevant antibodies for 20 minutes at room temperature in a dark room. After staining, erythrocytes were lysed by addition of 1 mL lysing solution (155 mM ammonium chloride, 0.1 mM sodium-EDTA, 10 mM potassium bicarbonate, pH 7.3, Ampliqon, Denmark), vortex mixed and incubated for 15 minutes at room temperature in a dark room. Samples were then analysed immediately by flow cytometry (BD FACS Canto II, Becton Dickinson) and data were analysed using FlowJo 9.2 software (FlowJo LLC). Cellular debris was excluded in a FSC/SSC plot and granulocytes excluded in a subsequent CD16/SSC plot. Monocytes were selected based on their distinct FSC/SSC scatter profile. Finally, monocyte subsets were identified according to the surface expression pattern of the lipopolysaccharide receptor CD14 and the Fcγ receptor CD16 and gated as classical (CD14⁺⁺CD16⁻),

non-classical (CD14⁺CD16⁺⁺) and intermediate (CD14⁺⁺CD16⁺) monocytes (Supplemental Figure 1). This gating strategy excluded CD16⁺ NK cells, which could otherwise have been interpreted as non-classical monocytes (exclusion of NK cells was verified in separate, direct staining experiments including anti-CD56 antibodies). Gating of monocyte subtypes was performed blinded to the ECR programs.

Statistical analyses

At baseline, continuous variables are given as mean ± standard deviation. Numerical baseline data were compared using the t-test. Before data analysis, all data were tested for the assumption of normality. Non-normally distributed numerical data were compared by the Wilcoxon signed-rank test. Data was furthermore analyzed in a mixed model for repeated measurements (ANOVA) taking into account different random variation over time. An inspection of the residuals and fitted values did not give rise to doubt the model. Analyses were performed on a log scale. Post hoc tests were made based on the Kenward-Roger approximation of the degrees of freedom. The differences between groups were compared using the ratio of medians and relative differences presented with 95% Confidence Intervals (CI). A complete intention to-treat analysis could not be performed, due to missing outcome data from patients who dropped out of the ECR program and blood sampling. A *p*-value below 0.05 was considered statistically significant. Statistical analyses were performed according to a modified intention to-treat principle using the *R* software (version 2.15.0), and Stata 15 (Stata Corp, College Station, TX).

Results

Baseline demographic and clinical characteristics

A total of 110 patients with IHD and referred to outpatient ECR were enrolled in the study, randomized to either the 8-week conventional or the optimized 12-week ECR program; thus, 56 patients were allocated to the conventional program and 54 patients to the extended program (Figure 1). Of these, 12 patients attended less than 75% of the training sessions. The patients not fully adhering to the ECR programs were evenly distributed between the two groups. Eleven patients were lost to follow-up. The baseline characteristics of the randomized patients are summarized in (Table 1). There were no significant differences in age, gender, BMI, or smoking habits. About four times as many men as women were referred to the ECR. The majority of the patients received statins as cholesterol-lowering treatment, and about half of the patients were in the polypharmacy group, defined as taking more than five prescribed drugs daily. Almost 70% of the patients were hypertensive and received medication for vascular disease. This medication included β-blockers, statins, glyceryl titrates, ACE-inhibitors, diuretics and anticoagulants. Remarkably, the monocyte concentration for both the 8- and the 12-week groups was in the high end of the reference interval for B-monocytes (0.2–0.7 x 10⁹/L) at baseline (Table 2). A total of 42% of the patients in the 8-week group and 46% of the patients in the 12-week group had a monocyte count higher than the reference level (above 700 per µL). However, at baseline there was no significant difference in absolute monocyte number between the groups. Those attending the 12-week program had a significantly higher absolute WBC count at baseline as shown in (Table 2). There were no outliers for sVCAM-1 in both groups.

Within-group comparison of inflammatory biomarkers from baseline to end of ECR

The group of patients participating in the optimized ECR

Table 3: Differences in raw medians for inflammatory biomarkers from baseline to end of exercise-based cardiac rehabilitation between the 12-week and 8-week groups.

	Baseline Median [25-75 quartiles]	End Exercise-based CR Median [25-75 quartiles]	p-value
sVCAM (ng/L)			
12-week n=42	682 [594.2; 779]	654.6 [564.3; 752.8]	p = 0.2790
8-week n=41	609.5 [522.8; 740.9]	611.4 [538.2; 767.8]	
IL-6 (ng/L)			
12-week n=45	4.0 [2.6; 5.0]	2.4 [1.8; 3.3]	p = 0.002
8-week n=42	3.6 [2.4; 5.6]	3.0 [1.7; 4.5]	
IL-6 (ng/L)a			
12-week n=42	3.8 [2.6; 4.7]	2.4 [1.8; 2.9]	p < 0.00001
8-week n=38	3.1 [2.3; 5.2]	2.9 [1.7; 4.0]	
hsCRP (mg/L)			
12-week n= 45	2.2 [1.1; 5.1]	1.3 [0.6; 2.6]	p < 0.00001
8-week n= 42	2.1 [0.8; 6.2]	1.3 [0.6; 2.5]	
hsCRP (mg/L)b			
12-week n= 39	2.2 [1.1; 4]	1.2 [0.6; 2.3]	p < 0.00001
8-week n= 37	1.8 [0.8; 3.6]	1.0 [0.6; 2]	
WBC (1/ μ L)			
12-week n=45	6760 [6080; 8510]	6330 [5600; 7560]	p= 0.0671
8-week n=42	6760 [5110; 7400]	6370 [5340; 7180]	
Monocytes (1/ μ L) Sysmex			
12-week n=45	600 [500; 800]	600 [460; 800]	p= 0.4370
8-week n=42	600 [500; 800]	600 [500; 800]	
CD14 ⁺⁺ CD16 ⁻ (/ μ L)			
12-week n=45	535.5 [432.3; 650.5]	488.3 [387.5; 604.6]	p= 0.0178
8-week n=42	480 [395.8; 693.5]	459 [388.6; 583.3]	
CD14 ⁺⁺ CD16 ⁺ (/ μ L)			
12-week n=45	24.1 [16.3; 34.6]	21 [15.2; 30.6]	p= 0.0683
8-week n=42	22.7 [12; 28.5]	18.5 [12.8; 26.5]	
CD14 ⁺ CD16 ⁺⁺ (/ μ L)			
12-week n=45	58.1 [35.9; 82.9]	58.5 [43.7; 74.7]	p= 0.1145
8-week n=42	44.7 [34.7; 63.3]	54.2 [36; 66]	

CD14⁺⁺CD16⁻: classical monocytes; CD14⁺⁺CD16⁺: intermediate monocytes; CD14⁺CD16⁺⁺: non-classical monocytes

a concentrations above 10 ng/L removed

b concentrations above 8 mg/L removed

program showed a significant decrease in hsCRP ($p = 0.0004$), the IL-6 concentration ($p = 0.0002$), the sVCAM-1 concentration ($p = 0.0362$) and in the monocyte count ($p = 0.0362$) (Figure 2), following the 12-week extended ECR. No significant change was observed for absolute WBC count ($p = 0.0516$). The group of patients participating in the 8-week conventional ECR showed a significant decrease in hsCRP ($p = 0.0027$) (Figure 2). Furthermore, no significant change was observed for IL-6 ($p = 0.0558$), sVCAM-1 ($p = 0.1968$), total monocyte count ($p = 0.3661$) or WBC ($p = 0.795$).

Differences between groups of inflammatory biomarkers from baseline to end of ECR

Concerning the primary outcome, the group of patients

participating in the optimized ECR program, showed a significant decrease in the sVCAM-1 concentration ($p=0.0362$) from baseline to end of ECR (Figure 2). However, the optimized ECR did not reduce the sVCAM-1 concentration more than the conventional ECR ($p=0.2790$) (Table 3). For the secondary outcomes, IL-6, hsCRP and the classical monocytes were significantly more reduced in the optimized group compared to the conventional group, ($p=0.002$), ($p<0.00001$) and ($p=0.0178$), respectively. Removing outliers for IL-6 and hsCRP did not seem to change the results (Table 3).

Differences between groups of inflammatory biomarkers over time

In the analyses of differences between the 8-week and 12-week

ECR programs, there were no overall differences over time concerning the primary or secondary outcomes as shown in (Table 3). However, there seemed to be a significant difference in hsCRP ($p=0.022$), IL-6 ($p=0.028$) and classical monocytes ($p=0.035$) at the 6-month follow-up in favor of the 12-week group (in all cases with wide 95% CI). This difference was not sustained at the 1-year follow-up (Table 2).

Discussion

To our knowledge, this is the first study to investigate the dose-response relationship between the frequency and duration of ECR programs and the reduction in low-grade vascular inflammation among patients with stable IHD.

We found that the optimized 12-week ECR program significantly decreased sVCAM-1, IL-6, hsCRP, monocytes and the intermediate CD14⁺⁺CD16⁺ subset. Participation in the conventional 8-week ECR program resulted only in a significant reduction of hsCRP and an increase in the non-classical CD14⁺CD16⁺ subset. Between groups analyses from baseline to the end of ECR showed no significant difference in the primary outcome sVCAM-1. However, IL-6, hsCRP and classical monocytes decreased significantly more in the optimized 12-week group compared to the conventional 8-week group (Table 3). Even though we observed a tendency towards a reduction in more inflammatory markers over time, this was not significant at 1-year follow up for any of the inflammatory biomarkers. We speculate that the lack of significant effects over time may be caused by the power calculations for the study being related to the VO_{2peak} measurements. Thus, this study may be underpowered for the biomarker measurements. In addition, IL-6 and hsCRP were reduced significantly more in the optimized program group of the ECR. Unfortunately, this pattern was not sustained at one-year follow up. We speculate that this could be caused by a decreased exercise effort over time. Unfortunately, we do not have data on this. However, due to the result at 1-year follow-up, it is likely that patients do not maintain the level of physical exercise after completion of even an optimized 12-week ECR program. To obtain a long-term reduction in other inflammatory markers, a supplementary supervised telemedicine solution after ending the hospital-based ECR program may contribute to integrate and sustain physical exercise as a part of daily life. Multiple factors in the human body influence inflammation. To study dose-response effects of exercise-based physical training it is crucial to have comparable study populations and sensitive and reliable assays to determine concentrations of the biomarkers. Proper randomization of the two groups in this study was ensured (Table 1). The majority of the included patients with IHD in both ECR groups received statin treatment (Table 1). Almost all of the patients received lipophilic statin treatment, and for the vast majority, a standard dose of 40mg/day Simvastatin. Besides lowering of cholesterol, statins possess a number of pleotropic effects, including inhibition of inflammatory cytokines. Some studies have suggested that statin treatment reduces inflammation and lowers the serum concentration of IL-6 and hsCRP [28-30]; however, some inconsistencies are reported in the literature. A recent Meta analysis states that especially the lipophilic statins reduce serum concentration of for example IL-6 and hsCRP and thereby decrease inflammation [31]. Thus, in our study it is likely that statin treatment also may have contributed to the reduction of the plasma concentration of IL-6 and CRP following ECR; but due to randomization, equally distributed in the two ECR groups. Additionally, Simvastatin treatment was initiated at least four to six weeks before enrolment into the ECR programs. Therefore, our

result of a significant decrease in hsCRP and IL-6 plasma concentrations after completion of ECR and at 1-year follow-up is most likely due to the completion of the optimized 12-week ECR program. Thus, it is possible that participating in an ECR program further contributes to the decrease in inflammatory cytokines and thereby reduction of arterial inflammation [32]. Previous studies based on similar interventions like the present study support that a 12-week ECR program with exercise three times per week results in a significant reduction of serum concentration of IL-6 and CRP at the highly sensitive level [7,33]. Even though most of our study participants were taking statins when enrolled in the study according to the hsCRP plasma concentration, 41% of the total number of included patients remained in the high-risk category (>3 mg/L). After completion of the extended 12-weeks ECR, only 18% remained in the high-risk category, indicating a beneficial effect of the optimized program (results not shown). Monocyte subset distribution seemed not to be associated with statin treatment, since it has been shown to be independent of statin treatment dose [34]. A recent study reporting the effect of two weeks cessation of statin therapy showed no significant difference in any monocyte subsets [35]. Intensively studied inflammatory biomarkers include sVCAM-1, the acute phase reactant hsCRP, the inflammatory cytokine IL-6, and monocyte count including sub typing. Stable plaque in the arteries is associated with lower levels of the above-mentioned parameters [36]. A major finding in this study is that an optimized 12-week supervised ECR program resulted in a significant decrease in inflammatory markers, including sVCAM-1, IL-6, hsCRP, monocytes (Figure 2) and the intermediate CD14⁺⁺CD16⁺ subset. Participation in the conventional 8-week ECR program resulted only in a significant reduction in hsCRP and an increase in the non-classic CD14⁺CD16⁺ subset, indicating that the optimized 12-week ERC program is more beneficial in reducing key markers of inflammation compared to the conventional program. As IL-6 is a primary driver of CRP, it was unexpected that only hsCRP showed a significant reduction in the conventional program. However, CRP is an important actor in atherosclerosis showing pro-atherogenic effects mainly by causing endothelial dysfunction [12]. Evidence shows that hsCRP decreases in response to exercise [7] and low levels of hsCRP are associated with a decreased relative risk of cardiovascular events [37]. Acknowledging hsCRP as an important inflammatory biomarker and predictor of cardiovascular disease, the significant decrease in hsCRP concentration during the 8-week ECR program suggests that the short exercise program is beneficial for the patients, too, although the effect was not as pronounced as in the 12-week program. Palmefors et al. [7] 2014 has reviewed the literature on the effect of physical exercise training on key inflammatory markers [7]. They found that the effect of physical exercise on specific inflammatory markers, including IL-6 and hsCRP, seemed to depend on the type, duration and intensity of the exercise intervention. The results of our study support this statement, as the optimized 12-week ECR program resulted in significant reductions in more key inflammatory markers compared to the conventional 8-week ECR program. Chronic inflammation results in systemic monocytosis which is linked to inflammatory activity in human atherosclerotic plaques [38]. With a mean of $645 \times 10^6/L$ for the 8-week group and $719 \times 10^6/L$ for the 12-week group, the majority of the patients included in the study were at the high end of the reference level [$200-700 \times 10^6/L$]. Within-group comparison showed a significant reduction of the total monocyte count; despite the reduction, the relative high mean concentrations persisted even at the one year follow-up. It seemed that the group of

patients with heart disease in this study had a high level of inflammation. Neither statin treatment nor ECR reduced the systemic monocytoysis, thus further investigations of potential monocytoysis reducing factors are needed. For the optimized ECR group there was a higher tendency to a decrease in the classical CD14⁺⁺CD16⁻ subset both at the end of the ECR and at 6-month follow-up, compared to the conventional group (Tables 2,3). Furthermore, we observed at small though significant decrease in the intermediate subset for the optimized ERC group at the end of the ECR, and a small though significant increase for the non-classical subset for the conventional ECR group. This may indicate a shift in the monocyte profile towards a decrease in the classical CD14⁺⁺CD16⁻ subset during the ECR program. This clearly emphasizes the need for further investigations before drawing conclusions related to a possible shift in monocyte subsets (Tables 2,3). Furthermore, recent results have demonstrated that arterial macrophages show multilayered ancestry, and the macrophages derived from circulating blood monocytes only transiently reside as arterial macrophages [39]. These results support that further studies are needed to explore if circulating monocytes are potential and relevant biomarkers for low-grade inflammation in patients with IHD. Especially the intermediate subset is linked to a central role in early subclinical atherosclerosis and advanced cardiovascular disease [25,40], but quantification and characterization of this small subset is associated with technical difficulties, especially accurate gating and quantification in whole-blood samples. In general, reliable analyses of monocyte subsets are technically demanding and crucial for the analyses. It is important to use freshly drawn blood samples and a single platform lyse-no-wash assay to avoid activation or loss of monocytes before flow cytometry [41]. Additionally, it is crucial to use a gating strategy ensuring that granulocytes and CD16⁺ NK cells are excluded [41]. In our study, monocyte subpopulations were analyzed using fresh blood samples on the day of inclusion of each individual participant and the gating strategy was similar to previously published gating strategies (Supplemental (Figure 1)) [25,42]. The monocytes gated with our strategy were negative for CD86 and C56, which are cell markers for granulocytes and NK cells, respectively. Gene expression profiling of the three-monocyte subtypes has revealed specific genes that are highly expressed in each of the three subtypes in healthy persons [22]. Subjecting mononuclear cells, isolated from buffy-coats from healthy blood donors, in our gating strategy and gene expression analysis confirmed that the S100A12 gene was highly expressed in the classical CD14⁺⁺CD16⁻ subset. We also found that the C1QB gene was highly expressed in the non-classical CD14⁺CD16⁺⁺ subset, and finally that the SCD gene showed elevated expression in the intermediate CD14⁺⁺CD16⁺ subtype (Supplemental (Figure 2)). These results clearly support the chosen gating strategy. However, it is a general limitation that different conditions for sample handling, sample storage, and flow cytometric analyses are used in the existing published studies. We support the recommendation from Hristov et al. [41] 2014 of a standardized and technically sound protocol for monocyte sub typing approved by an expert committee [41]. Some limitations in the present study should be addressed. The rather small number of patients included, could result in omission of some subtle changes in inflammatory biomarkers. Furthermore, it is difficult to relate the data for the monocyte subset to the existing literature due to the lack of standardization of monocyte subset analyses [41,42]. It was not possible to perform a complete intention to treat analysis because it was impossible to collect blood samples from the patients dropping out of the study. During this study, we noticed that many

patients declined to participate in the study and about 20% did not complete the ECR program. It is likely that a significant proportion of these patients stopped exercising and thereby missed the beneficial effects of ECR. We investigated both the total group attending the ECR programs and separate calculations of those attending at least 75% of the ECR program. This did not change any conclusions. Qualitative studies are needed to explore why patients decline to participate or did not complete the ECR program.

Conclusions

In conclusion, on a short term basis an optimized 12-week ECR intervention reduced significantly more inflammatory biomarkers in comparison with a conventional 8-week ECR intervention. However, this effect was not sustained on a long-term basis, which could be due to the study being underpowered. Further studies on ECR program duration and frequency are needed to identify the optimal framework for an ECR program to reduce key inflammatory biomarkers in atherosclerosis.

Acknowledgments

The authors thank Sara Rose Newell for excellent technical assistance, Senior Lecturer Thomas Bendtsen for statistical assistance, and the staff at Department of Clinical Immunology, Aarhus University Hospital, Aarhus, Denmark for helping with sample preparation and flow cytometry.

References

1. Heran BS, Chen JM, Ebrahim S, Moxham T, Oldridge N, Rees K, et al. Exercise-based cardiac rehabilitation for coronary heart disease. *Cochrane Database Syst Rev.* 2011;(7):CD001800.
2. Anderson L, Oldridge N, Thompson DR, Zwisler AD, Rees K, Martin N, et al. Rehabilitation for Coronary Heart Disease: Cochrane Systematic Review and Meta-Analysis. *J Am Coll Cardiol.* 2016;67(1):1-12.
3. Anderson LJ, Taylor RS. Cardiac rehabilitation for people with heart disease: an overview of Cochrane systematic reviews. *Cochrane Database Syst Rev.* 2014;(12):CD011273.
4. Achttien RJ, Staal JB, van der Voort S, Kemps HMC, Koers H, Jongert MWA, et al. Exercise-based cardiac rehabilitation in patients with coronary heart disease: a practice guideline. *Neth Heart J.* 2013;21(10):429-38.
5. Libby P, Hansson GK. Inflammation and immunity in diseases of the arterial tree: players and layers. *Circulation Research.* 2015;116(2):307-11.
6. Peterson JC, Charlson ME, Wells MT, Altemus M. Depression, coronary artery disease, and physical activity: how much exercise is enough? *Clin Ther.* 2014;36(11):1518-30.
7. Palmefors H, DuttaRoy S, Rundqvist B, Börjesson M. The effect of physical activity or exercise on key biomarkers in atherosclerosis--a systematic review. *Atherosclerosis.* 2014;235(1):150-61.
8. Lim S-T, Min S-K, Kwon Y-C, Park S-K, Park H. Effects of intermittent exercise on biomarkers of cardiovascular risk in night shift workers. *Atherosclerosis.* 2015;242(1):186-90.
9. Hartman J, Frishman WH. Inflammation and atherosclerosis: a review of the role of interleukin-6 in the development of atherosclerosis and the potential for targeted drug therapy. *Cardiol Rev.* 2014;22(3):147-51.
10. Lindmark E, Diderholm E, Wallentin L, Siegbahn A. Relationship between interleukin 6 and mortality in patients with unstable coronary artery disease: effects of an early invasive or noninvasive strategy. *JAMA.* 2001;286(17):2107-13.
11. Su D, Li Z, Li X, Chen Y, Zhang Y, Ding D, et al. Association between serum interleukin-6 concentration and mortality in patients with coronary

- artery disease. *Mediators Inflamm.* 2013;2013:726178.
12. Fordjour PA, Wang Y, Shi Y, Agyemang K, Akinyi M, Zhang Q, et al. Possible mechanisms of C-reactive protein mediated acute myocardial infarction. *Eur J Pharmacol.* 2015;760:72-80.
 13. Eisen A, Benderly M, Behar S, Goldbourt U, Haim M. Inflammation and future risk of symptomatic heart failure in patients with stable coronary artery disease. *Am Heart J.* 2014;167(5):707-14.
 14. Zamani P, Schwartz GG, Olsson AG, Rifai N, Bao W, Libby P, et al. Inflammatory biomarkers, death, and recurrent nonfatal coronary events after an acute coronary syndrome in the MIRACL study. *J Am Heart Assoc.* 2013;2(1):e003103.
 15. Pearson TA, Mensah GA, Alexander RW, Anderson JL, Cannon RO, Criqui M, et al. Markers of inflammation and cardiovascular disease: application to clinical and public health practice: A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. *Circulation.* 2003;107(3):499-511.
 16. Packard RRS, Libby P. Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. *Clin Chem.* 2008;54(1):24-38.
 17. Yamamoto E, Sugiyama S, Hirata Y, Tokitsu T, Tabata N, Fujisue K, et al. Prognostic significance of circulating leukocyte subtype counts in patients with coronary artery disease. *Atherosclerosis.* 2016;255:210-6.
 18. Ghattas A, Griffiths HR, Devitt A, Lip GYH, Shantsila E. Monocytes in coronary artery disease and atherosclerosis: where are we now? *J Am Coll Cardiol.* 2013;62(17):1541-51.
 19. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood.* 2010;116(16):e74-80.
 20. Zawada AM, Rogacev KS, Schirmer SH, Sester M, Böhm M, Fliser D, et al. Monocyte heterogeneity in human cardiovascular disease. *Immunobiology.* 2012;217(12):1273-84.
 21. Belge K-U, Dayyani F, Horelt A, Siedlar M, Frankenberger M, Frankenberger B, et al. The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. *J Immunol.* 2002;168(7):3536-42.
 22. Wong KL, Tai JJ-Y, Wong W-C, Han H, Sem X, Yeap W-H, et al. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood.* 2011;118(5):e16-31.
 23. Zawada AM, Rogacev KS, Rotter B, Winter P, Marell R-R, Fliser D, et al. SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset. *Blood.* 2011;118(12):e50-61.
 24. Shantsila E, Tapp LD, Wrigley BJ, Pamukcu B, Apostolakis S, Montoro-García S, et al. Monocyte subsets in coronary artery disease and their associations with markers of inflammation and fibrinolysis. *Atherosclerosis.* 2014;234(1):4-10.
 25. Rogacev KS, Cremers B, Zawada AM, Seiler S, Binder N, Ege P, et al. CD14++CD16+ monocytes independently predict cardiovascular events: a cohort study of 951 patients referred for elective coronary angiography. *J Am Coll Cardiol.* 2012;60(16):1512-20.
 26. Cappellari R, D'Anna M, Bonora BM, Rigato M, Cignarella A, Avogaro A, et al. Shift of monocyte subsets along their continuum predicts cardiovascular outcomes. *Atherosclerosis.* 2017;266:95-102.
 27. Patel VK, Williams H, Li SCH, Fletcher JP, Medbury HJ. Monocyte inflammatory profile is specific for individuals and associated with altered blood lipid levels. *Atherosclerosis.* 2017;263:15-23.
 28. Greque GV, Serrano CV, Strunz CM, Soeiro A, Santos M, Pivateli F, et al. Preprocedural statin therapy, inflammation and myocardial injury in low-risk stable coronary artery disease patients submitted to coronary stent implantation. *Send to Catheter Cardiovasc Interv.* 2016;87(2):222-9.
 29. Kim YR, Park JH, Lee H-J, Pyun WB, Park S-H. The effect of doubling the statin dose on pro-inflammatory cytokine in patients with triple-vessel coronary artery disease. *Korean Circ J.* 2012;42(9):595-9.
 30. Sola S, Mir MQS, Lerakis S, Tandon N, Khan BV. Atorvastatin improves left ventricular systolic function and serum markers of inflammation in nonischemic heart failure. *J Am Coll Cardiol.* 2006;47(2):332-7.
 31. Bonsu KO, Reidpath DD, Kadirvelu A. Effects of Statin Treatment on Inflammation and Cardiac function in Heart Failure: An Adjusted Indirect Comparison Meta-analysis of Randomised Trials. *Cardiovasc Ther.* 2015;33(6):338-46.
 32. Carbone F, Montecucco F. Inflammation in arterial diseases. *IUBMB Life.* 2015;67(1):18-28.
 33. Beckie TM, Beckstead JW, Groer MW. The influence of cardiac rehabilitation on inflammation and metabolic syndrome in women with coronary heart disease. *J Cardiovasc Nurs.* 2010;25(1):52-60.
 34. Krychtiuk KA, Kastl SP, Pfaffenberger S, Lenz M, Hofbauer SL, Wonnerth A, et al. Association of Small Dense LDL Serum Levels and Circulating Monocyte Subsets in Stable Coronary Artery Disease. *PLoS One.* 2015;10(4):e0123367.
 35. Jaipersad AS, Shantsila E, Blann A, Lip GYH. The effect of statin therapy withdrawal on monocyte subsets. *Eur J Clin Invest.* 2013;43(12):1307-13.
 36. Puz P, Lasek-Bal A, Ziaja D, Kazibutowska Z, Ziaja K. Inflammatory markers in patients with internal carotid artery stenosis. *Arch Med Sci.* 2013;9(2):254-60.
 37. Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *N Engl J Med.* 2000;342(12):836-43.
 38. Dutta P, Nahrendorf M. Regulation and consequences of monocytois. *Immunol Rev.* 2014;262(1):167-78.
 39. Klapproth K, Lasitschka F, Rodewald H-R. Multilayered ancestry of arterial macrophages. *Nat Immunol.* 2016;17(2):117-8.
 40. Rogacev KS, Seiler S, Zawada AM, Reichart B, Herath E, Roth D, et al. CD14++CD16+ monocytes and cardiovascular outcome in patients with chronic kidney disease. *Eur Heart J.* 2011;32(1):84-92.
 41. Hristov M, Heine GH. Monocyte subsets in atherosclerosis. *Hamostaseologie.* 2014 Nov 14;35(1).
 42. Kaito M, Araya S-I, Gondo Y, Fujita M, Minato N, Nakanishi M, et al. Relevance of distinct monocyte subsets to clinical course of ischemic stroke patients. *PLoS One.* 2013;8(8):e69409.