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The Labile Iron Pool in Monocytes Reflects the Activity of the Atherosclerotic Process in Men With Chronic Cardiovascular Disease

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Summary

The study investigates the relationship between the labile iron pool (LIP) in circulating monocytes and markers of iron metabolism, inflammation, oxidative stress, endothelial dysfunction and arterial elasticity in patients with chronic cardiovascular disease and in healthy volunteers. The patients with a history of CVEs had significantly higher LIP values than did the control group (1.94±0.46 μM vs. 1.62±0.49 μM, $p=0.02$). Except for the leukocyte number (WBCs), the groups did not differ in other inflammatory markers (CRPus, CD 163, MPO, MMP-1). Similarly, there were no differences in the markers of endothelial dysfunction (ICAM, VCAM, E-selectin, vWF). The CVE group had higher pulse pressures, levels of markers of impaired arterial elasticity (AI, Young´s modulus, pulsatility, stiffness index), IMT values and ABI values. The LIP concentration was significantly correlated with the transferrin receptor/ferritin ratio, hepcidin levels, VFT content and the ABI and ET values. Patients with a history of CVE have significantly higher concentrations of iron in their intracellular LIP in circulating monocytes than do healthy controls. The independent and significant correlation of LIP with markers of the progression of atherosclerosis and arterial stiffness suggests LIP as a possible novel marker of atherosclerotic activity.

Key words

Iron overload • LIP • Monocytes • Inflammation • Arterial stiffness

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Introduction

Iron as a cardiovascular risk factor has been discussed since the early 1980s. Sullivan (1981) was apparently the first who proposed that iron overload contributes to atherosclerosis and promotes cardiovascular disease. The presence of redox-active iron as well as elevated expression of H- and L-ferritin in human atherosclerotic plaques further supports the iron hypothesis (Smith *et al.* 1995, Pang *et al.* 1996). The results of several human epidemiological studies strongly suggest an association of the body's iron stores with cardiovascular mortality (Lauffer *et al.* 1990) and higher risks of myocardial infarction (Salonen *et al.* 1992, Tuomainen *et al.* 1998) or peripheral vascular disease (Menke *et al.* 2009). Kiechl *et al.* suggested serum ferritin levels as an indicator of carotid atherosclerotic progression (Kiechl *et al.* 1997). In a previous study, we described a significant correlation of stored iron levels with asymptomatic carotid atherosclerosis in healthy men of primary prevention (Syrovatka *et al.* 2011).

Iron is an essential element in the human body that plays an important role in oxygen metabolism, electron transfer, and acts as a cofactor for many enzyme systems. Under physiological conditions, the body keeps a stable pool of iron. Most of the surplus iron is stored in hepatocytes and in the mononuclear phagocyte system (MPS). The MPS (monocytes, macrophages and their precursors) plays a major role in the recycling of the body's iron pool (Kong *et al.* 2008).

Inflammation is a characteristic feature of atherosclerosis. Macrophages are a major type of

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inflammatory cell and play a crucial role in the development and progression of the atheromatous plaque. M1 macrophages are present in human atherosclerotic lesions (Khallou-Laschet *et al.* 2010), where they produce proinflammatory cytokines (TNFα, IL-1β, IL-6) contributing to local inflammation and the progression and eventual destabilization of the plaque. During plaque hemorrhage, the pro-oxidant environment of atherosclerotic tissue promotes erythrocyte lysis and the accumulation of free hemoglobin, which, unless eliminated, may cause tissue damage via oxidative stress (Habib and Finn 2014).

Iron within the plaque undoubtedly modifies the immunoreactivity and metabolism of macrophages/monocytes. However, the molecular mechanisms underlying the iron homeostasis, metabolism and immune activity of the MPS are poorly understood. High iron stores are associated with activation of both humoral and cellular immunity (De Palma *et al.* 2003). Iron and iron-binding proteins modulate the functions of B and T lymphocytes, macrophages and natural killer cells (NK cells) (Feng *et al.* 1994, Kemp 1993, Bovlus 2003). Iron seems to have a direct impact on macrophage activation and on their transformation to foam cells (Yuan *et al.* 2004).

Accordingly, it seems evident that the iron content of macrophages has the potential to significantly affect the atherosclerotic process. Most of intracellular iron is located in ferritin, but some can exist in a loosely bound condition as the cytosolic labile iron pool (LIP) (Greenberg and Wintrobe *et al.* 1946, Jacobs 1997), which is thought to represent the redox-active iron capable of catalyzing the formation of oxygen free-radicals. The LIP is a highly exchangeable pool of iron ions that sits on the crossroads of the metabolic pathways of iron-containing compounds (Kruszewski 2003) and is associated with a diverse population of ligands, such as phosphates, carboxylates, polypeptides, and the polar groups of membrane phospholipids (Kakhlon *et al.* 2002).

Our long-standing scientific interest is focused on the role of the body’s iron pool in the pathogenesis of cardiovascular diseases. In this study, we investigate a possible relationship between the LIP in circulating monocytes and other parameters of iron metabolism, inflammation, endothelial dysfunction and arterial elasticity in patients with a history of cardiovascular disease and in healthy volunteers.

Materials and Methods

Patients and controls

A total of 51 men volunteers were recruited into the study. The cohort of volunteers with cardiovascular events (patients after AMI or stroke, at least 3 months after the last acute coronary syndrome, with peripheral arterial disease) were recruited from the patients registered in the Department of Preventive Cardiology. The control group of healthy individuals were recruited from the volunteers with no history of CVE to obtain age matched groups. All respondents provided written informed consent, and the study was approved by the ethics committee of the 3rd Medical Faculty, Charles University, Prague. Exclusion criteria were an age over 65 years; any acute or chronic inflammatory disease or hemorrhagic diathesis; current use of immunosuppressants, non-steroidal anti-inflammatory drugs or chelates; and any surgery or transfusions of blood derivatives within last 3 months. The medical history and detailed information about previous cardiovascular diseases were obtained. All participants filled out Baecke’s habitual questionnaire focused on dietary habits and physical activity (Baecke *et al.* 1982, Florindo *et al.* 2006). Anthropometric measurements included body height, weight, and waist and hip circumferences. The body mass index (BMI) and waist-to-hip ratio (WHR) were calculated. Resting blood pressure (systolic/diastolic), ankle-brachial index (ABI), pulse pressure (PP), and mean arterial pressure (MAP) were measured/calculated in all respondents. We measured the cardio-ankle vascular index (CAVI), augmentation index (AI), pre-ejection time (PEP), and ejection time (ET) using a VaSera VS-1500N system (Fukuda Denshi).

Ultrasound measurements

High-resolution B-mode ECG-gated ultrasonography of both common carotid arteries was performed in all subjects while in the supine position. The extracranial carotid arteries were examined bilaterally using a Philips iU22 ultrasound system equipped with a 7.5 MHz linear array transducer, and analyzed using QLAB Quantification software (Philips). The intima-media thickness (IMT) was measured in diastole on the far walls of the common carotid arteries at a distance of 10 mm from the carotid bulb after 10 min of rest (Stein *et al.* 2008). Young´s modulus (YEM), stiffness parameter β (SI), pulsatility index (PI) and

resistance index (RI) were calculated for an estimation of arterial elasticity (Cavallini *et al.* 1996, Mitsumura *et al.* 2014).

Transverse scanning was performed to measure the maximum visceral fat thickness (VFT) using a 3.5-MHz ultrasound probe. Measurements were obtained 1 cm above the umbilicus. VFT was defined as the distance between the anterior wall of the aorta and the internal face of the rectoabdominal muscle perpendicular to the aorta. Images were obtained immediately after a calm and complete expiration to avoid the influence of the respiratory status or abdominal wall tension (de Lucia Rolfe *et al.* 2010). Measurements were performed by the same person three times and averaged.

Biochemical parameters

Blood samples were collected from the subjects early in the morning after at least 10 h of fasting. The samples were sent to the Department of Laboratory Diagnostics FNKV for the analysis of routine parameters (lipid profile (total cholesterol (TC), triglycerides (TGs), high-density lipoprotein (HDL-C), apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), lipoprotein a (Lp(a)), blood count and differential, glucose (GLC), C-peptide, insulin (INS), urea, creatinine, bilirubin, uric acid, minerals (Na, K, Cl, Ca, P, Mg), liver enzymes (alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP)), and iron-associated parameters (Fe, binding capacity of Fe (TBIC), ceruloplasmin). For the isolation of circulating monocytes 3 samples (6 ml each) of blood were taken with K_3EDTA used as an anticoagulant. All efforts were made to minimize hemolysis. Within one hour, the blood samples were transported on ice to the laboratory where the isolation of monocytes for LIP measurement started immediately. Samples for the remaining analyses were immediately isolated by centrifugation and stored at -80 °C until further analysis. Ferritin (L- and H-chains), transferrin and circulating transferrin receptors were measured using a commercial ELISA kit from USCN (Cloud Clone Corp./Wuhan USCN Business Co., Ltd., Houston, TX 77084, USA); hepcidin, an ELISA kit from DRG (DRG Diagnostics GmbH, Marburg, Germany); oxidized low-density lipoprotein (OxLDL) and myeloperoxidase (MPO), a kit from Mercodia (Mercodia AB, Uppsala, Sweden); anti-oxLDL, a kit from Biomedica (Biomedica Medizinprodukte, GmbH, Vienna, Austria); matrix metalloproteinase-1 (MMP-1), a kit from RayBiotech (RayBiotech Inc., Norcross, GA, USA); intercellular adhesion molecule 1 (ICAM-1), a Platinum kit from eBioscience (Bender MedSystems GmbH, Vienna, Austria); vascular cell adhesion molecule 1 (VCAM-1) and CD163, a kit from R&D Systems (R&D Systems, Inc., Minneapolis, MN, USA); E-selectin, von Willebrand factor (vWF) and transforming growth factor beta 1 (TGF-1beta), a kit from Abcam (Abcam, Cambridge, UK); ultrasensitive C-reactive protein (CRPus), a kit a kit from Roche using a COBAS 8000 analyzer (Department of Laboratory Diagnostics FNKV); Cu, using F-AAS (flame atomic absorption spectrometry) (SPADIA LAB, a.s.). Low-density lipoprotein (LDL) was calculated and also measured using a kit from Roche/Hitachi (COBAS c, LDL-cholesterol plus, 2nd generation, COBAS 8000 analyzer) (Department of Laboratory Diagnostics FNKV).

The insulin resistance index (homeostasis model assessment of insulin resistance, HOMA-IR) was calculated using the HOMA2 glucose homeostasis model (Levy *et al.* 1998). The atherogenic index of the plasma was calculated as the log[triglycerides/high-density lipoprotein (HDL) cholesterol] (Dobiasova and Frohlich 2001).

Isolation of circulating monocytes, measurement of cytosolic LIP and cellular ferritin

Reagents and antibodies

OptiPrep™ (60 % (w/v) iodixanol in water) was obtained from Axis-Shield Density Gradient Media (Alere Technologies AS, Oslo, Norway). The PCV Packed Cell Volume Tubes and "easy read" measuring device were purchased from TPP AG (Trasadingen, Switzerland). Calcein and its acetoxymethyl (AM) ester were manufactured by Molecular Probes (Thermo Fisher Scientific, Waltham, MA). Salicylaldehyde isonicotinoyl hydrazone (SIH) was a generous gift from Prof. Prem Ponka, McGill University, Montreal, Canada. Mouse monoclonal antibody to CD14, biotin conjugated (1B-293-C100) and mouse monoclonal IgG1 isotype control, biotin conjugated (1B-632-C100) were obtained from EXBIO Praha (Vestec u Prahy, Czech Republic). The Vectastain Elite ABC kit and 3,3'-diaminobenzidine (DAB) substrate kit were provided by Vector Laboratories, Inc. (Burlingame, CA). The PVDF blotting membrane used was the Immobilon-P from Millipore (Billerica, MA). The β-actin rabbit monoclonal antibody, HRP conjugate (#5125), FTH1 (H-ferritin) rabbit

monoclonal antibody (#4393), and anti-rabbit IgG HRP-linked antibody (#7074) were from Cell Signaling Technology, Inc. (Danvers, MA). The WesternBrightTM Quantum chemiluminescent HRP substrate was obtained from Advansta Inc. (Menlo Park, CA). Sodium dodecyl sulfate (SDS) was purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany). Cell culture medium (RPMI 1640, Dutch modification, with 1 g/l $NaHCO_3$, 20 mM HEPES, without glutamine), bovine serum albumin (BSA), HEPES, dimethyl sulfoxide (DMSO) and CC/MountTM aqueous mounting medium and other chemicals used were from Sigma-Aldrich (St. Louis, MO).

Isolation of monocytes from peripheral blood

We adopted the flotation method developed by Graziani-Bowering *et al.* (1997) with modifications for use with whole blood (Isolation of a monocyte 2013). Typically, blood samples from two subjects were processed in parallel. Samples and all solutions were precooled to 4 °C and kept on ice throughout the procedure.

A 1.070 g/ml density barrier-solution was prepared by mixing 10 ml of OptiPrep^{TM} with 40 ml of RPMI 1640 and 0.5 % (w/v) BSA (RPMI-BSA) and 54 % (w/v) iodixanol was made by combining 7.2 ml of OptiPrepTM with 0.8 ml of 8.5 % (w/v) NaCl. Venous blood (16 ml) was gently mixed with 3.2 ml of the 54 % iodixanol and dispensed in 4 ml aliquots into 15-ml polypropylene tubes. 6 ml of the density barrier solution was carefully layered over the blood in each tube, followed by 0.5 ml of RPMI-BSA placed on top. The gradients were centrifuged at 700 g in a swinging bucket rotor for 30 min at 4 °C with slow deceleration. The monocytes were collected from the upper interface, diluted with 4 volumes of RPMI-BSA and centrifuged at 300 g for 10 min at 4 °C. The resulting cell pellet was resuspended in 2 ml of RPMI-BSA and kept on ice.

Nucleated elements were counted in a hemocytometer chamber with Türk's solution. The cell suspension was then used measure the LIP as described below. Smears were prepared for CD14 staining and the volume of cells was estimated using the PCV tubes. The remaining cells were denatured for Western immunoblotting by mixing 4 volumes of the cell suspension with 1 volume of 50 g/l SDS and 0.5 M dithiothreitol and boiling for 10 min.

The isolation procedure described above reproducibly yielded a suspension of fully viable mononuclear cells, free from granulocytes and platelets. On average, 2.2×10^6 cells were obtained from 10 ml of blood. According to CD14 staining, the mean purity was 56 %; the other cells appeared to be lymphocytes. The average recovery was 22 % relative to the original number of monocytes in the blood samples. Erythrocytes were never found in the preparations. However, a quantitatively minor (<10 %) contamination with fragments of red blood cells was present in about half of the cases, visible as a reddish upper rim on the cells pelleted in the PCV columns.

Measurement of the LIP in isolated monocytes

The concentration of the LIP in isolated monocytes was measured according to Epsztejn *et al.* (1997). Briefly, 10^6 isolated cells in 2 ml RPMI-BSA were loaded with 0.25 µM calcein AM ester for 5 min at 37 °C, resuspended in RPMI-BSA, and transferred to 4 ml of HEPES-buffered saline (20 mM HEPES/NaOH at pH 7.3 and 150 mM NaCl). Three milliliters of the cell suspension in a spectrofluorimetric quartz cuvette with magnetic stirrer were placed in the thermostated cuvette holder of a Fluoromax 3 spectrophotometer (HORIBA Jobin-Yvon S.A.S., France). Fluorescence of the cell suspension was monitored at an excitation wavelength of 488 nm and emission wavelength of 510 nm with constant stirring at 25 °C. The background signal was recorded for 400 s (F1), followed by measurement with 100 µM of SIH for 300 s (F2), and with a suitable amount of a standard solution of calcein for another 200 s (F3). The standard was prepared by dissolving calcein (free acid) in 50 mM K_2HPO_4 to approximately 1.6 µM. The exact concentration was determined spectrophotometrically (ε=77,000 cm^{-1} M^{-1} at 494 nm, based on the manufacturer's data). The standard was aliquoted and stored at -20 °C and carefully protected from light; new aliquots was used on each measurement day.

The mean values of F1, F2 and F3 were obtained from the last 150 s of each trace and used to calculate the LIP (µM) according to Epsztejn *et al.* (1997), including the dissociation constant of the iron-calcein complex (K_d=0.22 µM). The cell volume needed for the calculation was measured using the PCV columns.

Immunocytochemical staining for CD14

The smears of isolated cells on glass slides were fixed in freshly prepared ice-cold 3.7 % (v/v) formaldehyde in phosphate-buffered saline (PBS) for 10 min. The slides were blocked in 1 g/l BSA in PBS

(PBS-BSA) for 30 min and incubated with either an antibody against CD14 or an IgG1 isotype control antibody (negative control) at a 1:500 dilution in PBS-BSA for 30 min. The slides were then developed with the ABC reagent and the DAB substrate as directed by the manufacturer and, finally, mounted with CC/MountTM mounting medium.

The stained smears were examined with an inverted microscope equipped with a digital camera. Three random digital images were captured at 200× in both phase contrast and bright field modes and used to count the cells to determine the mean proportion of CD14+ cells (purity) for each preparation.

Western immunoblotting for ferritin in isolated monocytes

The samples of SDS-denatured monocytes were applied to 4.5 %-12.5 % SDS-polyacrylamide gels, together with a standard prepared by mixing several samples with approximately average cell concentrations. The proteins were resolved via electrophoresis (15 mA/gel) and transferred onto a PVDF blotting membrane. The blots were blocked for one hour with 5 % (w/v) skim milk in Tris-buffered saline with 0.05 % (w/v) Tween 20 (TBST) and then incubated with antibodies against human H-ferritin at a 1:1,000 dilution in 5 % (w/v) skim milk in TBST overnight at 4 °C on a tilting platform, followed by secondary an incubation with HRP-conjugated anti-rabbit antibody at a dilution of 1:1,000 in 1 % (w/v) skim milk in TBST for one hour at room temperature. The blots were then washed with TBST and developed with the WesternBright^{TM} Quantum chemiluminescent HRP substrate, as directed by the manufacturer. The chemiluminescence was read using a Fusion FX7 instrument (Vilber Lourmat, Marne-la-Vallée, France) and evaluated with Vilber Lourmat Bio-1D software version 15.03. The volumes of the bands were obtained from an optimally exposed picture and normalized to the signal of the standard present in each gel. The blots were then subjected to another detection with the HRP-linked antibody against human β-actin at a dilution of 1:1,000 in 5 % (w/v) skim milk in TBST. Initially, 6 µl (30 µg of BSA) of each sample was applied; the results were used to calculate the equivalent sample amounts and all samples were re-assayed. Both the H-ferritin and actin signals were normalized to the standard present in each gel, and for each sample, values are reported as the H-ferritin/actin ratio.

Statistical analysis

Statistical analyses were performed by a professional statistician using Statistica 12.0 (StatSoft, USA). Comparison of the groups was performed using Student's *t*-test for two independent samples. For variables with a nonhomogeneous distribution, the Mann-Whitney U test was used. Pearson's or Spearman's correlation analyses were used to assess any potential relationships between the LIP and other variables. Statistical significance was defined as $P<0.05$.

Results

The basic characteristics of the volunteers are shown in Table 1 (A, B, C, D). The groups did not differ in basic characteristics, such as age, weight, BMI or waist circumference, but CVE patients had thicker visceral fat tissue. The CVE patient group also had a significantly higher pulse pressure, although no differences in blood pressure were observed (Table 1A). For the basic biochemical parameters, we found significantly lower concentrations of TC, LDL, and apo-B in the CVE group than in the control group. CVE patients had higher concentrations of TGs and lower concentrations of HDL than the healthy volunteers. Moreover, there were also significantly higher levels of plasma glucose, C-peptide and insulin and a higher calculated insulin resistance index (HOMA-IR) in the CVE patient group (Table 1B). The patients with a history of CVE demonstrated significantly higher values for the LIP even though there was no difference in other markers of iron metabolism. Additionally, CVE individuals had higher concentrations of copper and ceruloplasmin than the healthy individuals (Table 1C).

Except for the number of leukocytes (WBCs), there was no difference in the other measured inflammatory parameters (CRPus, CD 163, MPO, MMP-1) between the groups. Similarly, the cohorts did not differ in the common markers of endothelial dysfunction (ICAM, VCAM, E-selectin, vWF) or in the parameters related to LDL oxidation. However, a significantly lower level of anti-oxLDL antibodies in CVE patients was found (Table 2).

As expected, the CVE patient group showed higher levels of markers of impaired arterial elasticity (AI, Young´s modulus, PI, SI) markers of atherosclerotic progression (IMT, ABI). The CVE patients also had thicker visceral fat measured by ultrasound (Table 3).

Because of the significant differences in the

concentrations of the LIP, we calculated the correlation of the LIP with other measured parameters. Of the factors related to iron metabolism, the LIP correlated significantly with the TfR/F ratio and with hepcidin. Of the measured inflammatory parameters, the LIP correlated only with the number of WBCs. Nevertheless, the LIP showed a significant correlation with ABI, the marker of atherosclerotic progression, and ET, the parameter of arterial stiffness. Interestingly, the LIP was positively correlated with VFT (Table 4).

Multivariate regression analysis confirmed that each correlation of the LIP was independent of the other correlated parameters except for LIP´s correlation with the VFT (Table 5).

Further analysis showed that the OR for acute CVE was 6 fold higher when LIP tertiles were compared (OR=5.96, P=0.0156) (Table 6).

Table 1. The basic characteristics of the volunteers (**A**, **B**). Basic biochemical parameters (**C**). Parameters of iron and copper metabolism (**D**).

A)

	Healthy group		CVE group		P
	n	x±SD	n	x±SD	
Age (years)	28	54.56±6.62	23	57.27±5.44	0.1223
Weight (kg)	28	88.30±18.00	23	91.83±15.38	0.4613
BMI (kg/m^2)	28	27.93±4.67	23	29.95±4.81	0.1357
Waist (cm)	27	96.50±13.75	23	102.15±11.52	0.1255
Hips (cm)	27	101.44±11.43	23	101.70±7.79	0.9293
Visceral fat thick. (cm)	28	6.13±2.22	23	8.52±2.86	0.0015
FAT subcut. (cm)	28	2.40±0.84	23	2.18±1.00	0.3994
Systolic BP (mmHg)	28	132.96±19.00	23	141.62±11.75	0.0627
Diastolic BP (mmHg)	28	87.14±14.67	23	88.47±8.62	0.7033
Pulse pressure	28	45.8±7.10	23	53.15±8.53	0.0016

B)

Diseases, risk factors	**Healthy group (28 men)**		**CVE group (23 men)**	
	Num. of cases	%	Num. of cases	%
History of stroke	0	0.0	1	4.3
History of AIM	0	0.0	14	60.9
Peripheral arterial disease	0	0.0	10	43.5
Ischemic heart disease	0	0.0	17	73.9
Effort angina pectoris	0	0.0	2	8.7
Hypertension	6	21.4	16	69.6
Diabetes mellitus 1. type	0	0.0	0	0.0
Diabetes mellitus 2. type	1	3.6	10	43.5
Hyperlipidemia	4	14.3	18	78.3
Active smokers	21	75.0	4	17.4
Former smokers	2	7.1	9	39.1
Positive FH for CVE	4	14.3	4	17.4
Medication				
Antihypertensives				
BB	2	7.1	16	69.6

CCB	2	7.1	6	26.1
ACE/ARB	3	10.7	17	73.9
Diuretics	0	0	7	30.4
Others	3	10.7	3	13.0
Antiplatelet agents	0	0	18	78.3
Lipid lowering drugs				
Statins	3	10.7	23	100
Fibrates	0	0	5	21.7
Ezetimib	0	0	7	30.4
Antidiabetics				
PAD	1	3.6	7	30.4
Insulin	0	0	1	4.3

C)

	Healthy group		**CVE group**		**P**
	N	x±SD	N	x±SD	
Urea (mmol/l)	28	5.25±1.15	23	5.57±1.45	0.3887
Creatinine (µmol/l)	28	92.6±11.86	23	88.5±13.19	0.2493
Uric acid (µmol/l)	28	332.6±72.33	23	339.5±79.86	0.7475
ALT (µkat/l)	28	0.54± 0.22	23	0.88±1.69	0.2937
AST (µkat/l)	28	0.49±0.13	23	0.63±0.96	0.4653
GMT (µkat/l)	28	0.50±0.34	23	0.87±0.54	0.0045
ALP (µkat/l)	28	1.09±0.28	23	1.25±0.42	0.1158
Bilirubin (µkat/l)	28	12.2±7.27	23	10.39±9.11	0.4410
Total cholesterol (mmol/l)	28	5.13±0.58	23	4.18±0.86	0.0001
HDL (mmol/l)	28	1.38±0.36	23	1.15±0.39	0.0389
LDL (mmol/l) – estim.	28	3.54±0.62	23	2.39±0.71	0.0001
LDL (mmol/l) – calcul.	28	3.15±0.65	23	2.17±0.69	0.0001
Triglycerides (mmol/l)	28	1.33±0.55	23	1.88±1.00	0.0176
Apo A1 (g/l)	28	1.56±0.24	23	1.51±0.30	0.5128
Apo B (g/l)	28	1.05±0.16	23	0.91±0.20	0.0094
Lp(a) (g/l)	28	22.4±63.22	23	55.7±84.64	0.1144
Glucose (mmol/l)	28	5.08±0.99	23	7.31±2.85	0.0003
C-peptide (pmol/l)	28	564.6±232.77	23	773.5±337.07	0.0120
Insulin (mU/l)	28	10.1±6.40	23	17.8±8.47	0.0006
WBCs ($x10^9$*/l)*	28	5.80±1.75	23	7.77±2.02	0.0005
RBC ($x10^{12}$*/l)*	28	4.86±0.30	23	4.91±0.36	0.5800
HGB (g/l)	28	146.8±10.86	23	152.8±12.95	0.0779
HCT (l)	28	0.43±0.02	23	0.44±0.03	0.1507
PLT ($x10^9$*/l)*	28	220.2±46.45	23	228.8±60.43	0.5696
NE (%)	28	56.1±9.17	23	57.1±13.98	0.7650
LY (%)	28	31.4±7.83	23	27.0±9.41	0.0771
MO (%)	28	7.75±2.18	23	8.50±3.07	0.3156
HOMA IR	28	2.35±1.65	23	5.96±3.87	0.0001

D)

	Healthy group		CVE group		P
	n	x±SD	n	x±SD	
Fe (μmol/l)	28	18.05±6.04	23	18.27±5.12	0.8971
TIBC (μmol/l)	28	54.43±9.80	23	58.22±7.92	0.1412
Ceruloplasmin (g/l)	28	0.21±0.02	23	0.23±0.03	0.0166
Cu (μmol/l)	26	13.99±1.94	22	15.64±2.34	0.0105
Ferritin (ng/ml)	25	129.09±122.90	22	102.30±75.88	0.3812
H-Ferritin (ng/ml)	25	26.75±22.03	22	24.12±18.42	0.6618
L-Ferritin (ng/ml)	25	10.49±3.13	22	9.12±2.37	0.1021
Transferrin (mg/ml)	25	7.78±2.65	22	8.13±3.08	0.6765
Transferrin rec.(ng/ml)	25	30.27±9.25	22	30.40±8.80	0.9587
Transferrin rec./Ferritin	25	0.54±1.03	22	0.45±0.39	0.6938
Hepcidin (ng/ml)	25	18.35±5.75	22	17.71±6.18	0.7169
LIP (μM)	28	1.62±0.49	23	1.94±0.46	0.0200
Cellular ferritin (H-Ferritin/Actin)	28	1.25±1.75	23	1.41±1.44	0.7257
Yield of monocytes (%)	28	0.24±0.13	23	0.23±0.05	0.5499

Table 2. Parameters of inflammation, endothelial dysfunction, oxidative stress.

	Healthy group		CVE group		P
	n	x±SD	n	x±SD	
CRP us (mg/l)	28	1.62±1.397	23	2.54±3.222	0.1755
CD163 (ng/ml)	25	592.6±158.39	22	647.4±257.51	0.3781
MPO (μg/l)	25	75.3±115.31	22	56.5±14.13	0.4531
MMP-1 (pg/ml)	25	621.7±722.6	22	940.4±963.6	0.2029
ICAM-1 (ng/ml)	25	239.1±77.74	22	273.4±59.35	0.0992
VCAM (ng/ml)	25	841.5±205.77	22	815.1±289.38	0.7170
vWF (mU/ml)	25	1050.8±464.31	22	901.1±519.49	0.3046
E-selectin (ng/ml)	25	9.17±4.15	22	10.7±5.85	0.3117
oxLDL (U/l)	25	27.4±8.50	22	27.0±7.79	0.8529
Anti-oxLDL (mU/ml)	25	497.3±489.59	22	245.0±292.60	0.0405

Table 3. Parameters of arterial elasticity and atherosclerotic progression.

	Healthy group		CVE group		P
	n	x±SD	n	x±SD	
PI	28	1.67±0.322	23	2.024±0.591	0.0098
RI	28	0.712±0.052	23	0.743±0.069	0.0800
SI	28	7.00±2.900	23	10.29±3.768	0.0010
YEM	28	1399.3±658.4	23	1875.2±791.4	0.0231
CAVI	28	8.00±1.018	23	8.47±1.219	0.1418
AI	28	1.04±0.188	23	1.26±0.309	0.0028
ET	28	301.9±21.00	23	294.5±29.58	0.3000
PEP	28	97.1±23.31	23	105.9±22.52	0.1777

PEP/ET	28	0.324±0.070	23	0.364±0.100	0.0945
ABI	28	1.17±0.067	23	0.951±0.155	0.0001
IMT (mm)	28	0.560±0.097	23	0.648±0.148	0.0144

Table 4. Univariate regression analysis of LIP's correlation with the measured parameters.

Variable	R	Variable	R
Age	-0.1220	*H-Ferritin*	-0.1654
Weight	0.0895	*L-Ferritin*	-0.0664
BMI	0.1750	*H/L-Ferritin Ratio*	-0.2075
Waist	0.1892	*Ferritin*	0.2444
VFT	0.3288*	*Transf. rec./Ferritin Ratio*	-0.3261*
FAT subcut.	-0.0272	*Cellular ferritin (H-Ferritin/Actin)*	0.0099
Systolic BP	0.2576	*Yield of monocytes*	0.1575
Diastolic BP	0.1702	*MPO*	-0.0109
IMT	0.2032	*oxLDL*	0.0257
SI	0,2524	*Anti oxLDL*	-0.1055
YEM	0.2303	*Hepcidin*	0.3365*
CAVI	-0.0670	*ICAM-1*	-0.0587
ABI	-0.3377*	*MMP-1*	0.0883
AI	0.1674	*E-selectin*	0.1459
PEP	0.1393	*vWF*	-0.1787
ET	-0.2748*	*VCAM*	0.1241
Total cholesterol	0.0380	*CD163*	0.0948
HDL cholesterol	-0.1018	*Glucose*	0.1494
LDL cholesterol	0.0221	*Insulin*	0.0181
Triglycerides	0.2034	*Homa IR*	0.0770
Apo A1	0.0264	*CRPus*	0.1913
Apo B	0.0246	*WBCs*	0.2791*
Lp(a)	0.0520	*HGB*	0.1460
PP	0.2443	*HCT*	0.1142
Fe	0.1371	*Baecke score – free time*	-0.2811*
TIBC	-0.1789	*Baecke score – work time*	-0.0201
Ceruloplasmin	0.0442	*Baecke score – household time*	-0.0119

* P<0.05

Table 5. Multivariate regression analysis of LIP with correlated factors.

	b	P
Total	1.4627	0.2584
ABI	-0.1094	0.8312
Baecke score – free time	-0.1240	0.2023
VFT	0.0809	0.0083
ET	0.0002	0.9505
Hepcidin	0.0080	0.5748
Transfer. Rec./Ferritin	-0.1697	0.0875
WBCs	0.0133	0.7090

Table 6. Prediction of acute CVE* by the LIP values.

	Upper tertile of LIP	Lower tertile of LIP	Total
CVE +	11	4	15
CVE -	6	13	19
Total	17	17	34
	OR=5.96, P=0.0156		

* Patients after AMI, stroke, or claudication.

Discussion

We found that patients with a history of CVE had significantly higher concentrations in their intracellular monocyte LIP than did healthy control individuals. The two groups did not statistically differ in the other estimated markers of iron metabolism; however, LIP showed a positive correlation with total body iron stores, expressed as the TfR/F ratio. Because plasma ferritin and transferrin are acute-phase proteins, TfR/F may be a better marker of total body iron stores (Tuomainen *et al.* 1998) and has already used in our previous studies (Syrovatka *et al.* 2011, Syrovatka *et al.* 2009). LIP was also positively correlated with plasma hepcidin. This critical regulatory protein of iron metabolism is produced mostly by hepatocytes in response to high body iron content. Hepcidin directly binds to ferroportin, a major iron export protein in hepatocytes, enterocytes and macrophages, leading to decreased iron absorption (blocked export from enterocytes) as well as the inhibition of iron release from hepatocytes and macrophages. Hepcidin is another acute phase protein, often elevated by inflammation. To the best of our knowledge, none of the subjects in our study showed any signs or had a history of acute or chronic inflammatory disease within the 3 months prior to their examination. The number of WBCs was the only marker of inflammation that differed between the CVE patients and the controls. Moreover, WBCs significantly correlated with the LIP, suggesting that WBC numbers remain a sensitive indicator of activated immunity, even in patients with clinically stable cardiovascular diseases.

The LIP showed significant and independent correlations with markers of atherosclerotic progression (ABI) and arterial stiffness (ET). Thus, it seems likely that redox-active iron in monocytes triggers signaling cascades mediated by proinflammatory cytokines (TNFα, IL-1β, IL-6) that affect other cells participating on the formation of atherosclerotic plaques (endothelial cells, smooth muscle cells, thrombocytes, T-lymphocytes). Increased intracellular concentrations in the LIP in monocytes may thus contribute to the perpetuation of the atherosclerotic process. As expected, no differences were found in any of the estimated parameters of endothelial dysfunction (ICAM, VCAM, E-selectin, vWF) between the groups. These markers have short plasma half-lives (several min) and are usually not elevated in patients with chronic cardiovascular diseases. (Pearson *et al.* 2003).

We are aware of methodological limitations imposed by our choice of the procedure for the isolation of circulating monocytes. The separation of monocytes from other peripheral blood cells is generally difficult. For the LIP measurement we looked for a method that would be rapid and avoid the activation of monocytes, contamination with platelets, and any magnetic fields, as well as the lysis of erythrocytes, which would liberate a large quantity of iron. The flotation method used fulfilled these criteria; however, it gave us preparations of monocytes that on average were quite heavily contaminated with lymphocytes and to some extent with fragmented erythrocytes as well. It should be noted that even if the mean purity was merely 56 %, the majority of the signal during the spectrofluorimetric LIP measurement, perhaps approximately 80 %, still came from monocytes because these cells are larger than lymphocytes. Perhaps of even bigger concern, the yield and purity were also quite variable, which in our belief reflected genuine differences in the specific gravity of cells obtained from different subjects. The cell recovery is included in Table 1D as 'Yield of monocytes (%)'. It is the number of isolated cells corrected for purity of each preparation as obtained from CD14 staining (% monocytes), expressed as percentage of the original number of monocytes in the blood sample according to blood count. It combines information about purity of each preparation and cell recovery into a single parameter.

Each subject was examined only once, so that we are no able to assess the variability of different runs in one donor. However, data from our pilot study focused on the isolation procedure demonstrated quite consistent results. Moreover, the LIP data displayed no statistical correlation with either the yield or the purity of the cell preparations, clearly indicating that our findings reported here are not due to experimental artifacts. A simple explanation might be that the differences in the LIP between the CVE patients and the controls might, in fact, reflect an abnormality in the cellular iron balance that is fairly general, occurring in various cell types, including lymphocytes and red blood cells. If this is the case, then the purity of the monocyte preparations is obviously not a significant issue. However, the same does not apply to the measurements of cellular ferritin by Western immunoblotting, where a statistically significant correlation with the yield and purity was observed, and any useful information in these data could be obscured by the heterogeneity of the cellular preparations.

The cohort of CVE patients had lower concentrations of plasma total cholesterol, LDL-cholesterol, and Apo B than were found in the healthy controls. This comes as no surprise because all CVE patients were on statin treatments. According to current guidelines (ESC/EAS Guidelines 2011, ACC/AHA Guideline 2014), therapy with statins is mandatory for all secondary prevention patients. This may also explain why no correlation of the LIP with plasma oxLDL was found. However, it should be added that the process of lipid oxidation occurs primarily in tissues, rather than in the plasma. Nevertheless, we found a correlation of iron stores with markers of both lipid as well as protein oxidation in our previous studies (Syrovatka *et al.* 2011, Syrovatka *et al.* 2009).

Patients with a history of CVE had significantly higher concentrations of plasma triglycerides and lower concentrations of HDL-cholesterol, indicating a higher incidence of metabolic syndrome than in the control group. Indeed, the CVE patients showed higher concentrations of plasma glucose, C-peptide, and insulin, as well as markers of insulin resistance (HOMA-IR). Although there was no difference in the waist circumference or BMI between the groups, significantly thicker VFT was found in the CVE patient cohort, as determined via precise ultrasound measurements of abdominal fat. Moreover, VFT was positively correlated with the LIP.

The correlation of elevated body iron stores with insulin resistance and obesity has been well known since 1992, when Salonen *et al.* published their first study on ferritin as a risk factor of acute myocardial infarction. This association has not yet been fully clarified. It is believed that increased iron consumption, namely heme iron in red meat, correlates with higher total energy intake levels in these individuals. On the other hand, heme iron may directly contribute to increased oxidative stress resulting in insulin resistance and obesity (Park *et al.* 2009). It has been demonstrated that oxidative stress in mammalian skeletal muscle leads to substantial insulin resistance due to impaired distal insulin signaling and glucose transport activity caused by a selective loss of the IRS-1 protein (Archuleta *et al.* 2009).

Until now it has not been fully elucidated which pathophysiological mechanism is mostly responsible for the LIP regulation in monocytes. Interventional studies using phlebotomy and/or reduced heme iron intake are needed to clarify whether elevated LIP levels could be effectively modified.

Conclusion

Patients with a history of CVE have significantly higher iron concentrations in the intracellular LIP of circulating monocytes than are found in healthy controls. No differences were found in other markers of iron metabolism. However, a regression analysis showed that the LIP positively correlated with TfR/F and plasma hepcidin, both markers of body iron stores. The LIP was also correlated with visceral fat tissue, which documents a possible role of iron in the development of insulin resistance. The significant and independent correlations of the LIP with markers of atherosclerotic progression (ABI) and arterial stiffness (ET) imply that the LIP could be a sensitive marker of atherosclerotic activity, even in patients with clinically stable cardiovascular diseases.

Conflict of Interest

There is no conflict of interest.

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