Exercise Training in Patients with Chronic Heart Failure Promotes Restoration of High-Density Lipoprotein Functional Properties

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Rationale: High-density lipoprotein (HDL) exerts endothelial-protective effects via stimulation of endothelial cell (EC) nitric oxide (NO) production. This function is impaired in patients with cardiovascular disease. Protective effects of exercise training (ET) on endothelial function have been demonstrated.

Objective: This study was performed to evaluate the impact of ET on HDL-mediated protective effects and the respective molecular pathways in patients with chronic heart failure (CHF).

Methods and Results: HDL was isolated from 16 healthy controls (HDL$_{\text{healthy}}$) and 16 patients with CHF-NYHA-III (HDL$_{\text{NYHA-III}}$) before and after ET, as well as from 8 patients with CHF-NYHA-II (HDL$_{\text{NYHA-II}}$). ECs were incubated with HDL, and phosphorylation of eNOS-Ser1177, eNOS-Thr495, PKC-βII-Ser660, and p70S6K-Ser411 was evaluated. HDL-bound malondialdehyde and HDL-induced NO production by EC were quantified. Endothelial function was assessed by flow-mediated dilatation. The proteome of HDL particles was profiled by shotgun LC-MS/MS. Incubation of EC with HDL$_{\text{NYHA-III}}$ triggered a lower stimulation of phosphorylation at eNOS-Ser1177 and a higher phosphorylation at eNOS-Thr495 when compared with HDL$_{\text{healthy}}$. This was associated with lower NO production of EC. In addition, an elevated activation of p70S6K, PKC-βII by HDL$_{\text{NYHA-III}}$ and a higher amount of malondialdehyde bound to HDL$_{\text{NYHA-III}}$ compared with HDL$_{\text{healthy}}$ was measured. In healthy individuals, ET had no effect on HDL function, whereas ET of CHF-NYHA-IIb significantly improved HDL function. A correlation between changes in HDL-induced NO production and flow-mediated dilatation improvement by ET was evident.

Conclusions: These results demonstrate that HDL function is impaired in CHF and that ET improved the HDL-mediated vascular effects. This may be one mechanism how ET exerts beneficial effects in CHF. (Circ Res. 2013;113:1345-1355.)

Key Words: aerobic exercise ■ eNOS ■ lipoproteins, HDL

An abnormality in endothelium-dependent vasodilation is a key phenomenon in patients with chronic heart failure (CHF). Numerous animal$^{1,2}$ and human$^{3,4}$ studies demonstrated a significant improvement in endothelium-dependent relaxation by exercise training (ET). An important factor responsible for this improvement is the increase in the bioavailability of NO. Studies using cultured endothelial cells$^{5}$ (ECs) or vessel tissue specimens obtained from trained animals$^{6}$ or humans$^{7}$ clearly documented that an increase in shear stress resulted in activation of endothelial NO synthase (eNOS).

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High-density lipoprotein (HDL) levels $>$40 to 60 mg/dL have been proposed as strong independent predictor of lower coronary artery disease risk.$^{8,9}$ Besides promotion of reverse cholesterol transport, HDL has been found to exert important antiatherogenic effects by stimulation of EC NO production and endothelial repair, as well as anti-inflammatory and antioxidant effects.$^{10-12}$ Recent studies revealed that the functional properties
of HDL with respect to stimulation of NO production are significantly impaired in patients with diabetes, coronary artery disease, and primary antiphospholipid syndrome. In these patients, malondialdehyde (MDA)-modified HDL may trigger the activation of PKC-β, thereby reducing eNOS-dependent NO production. These findings on the functional capacity of HDL in influencing endothelial function might promote a shift in therapeutic approaches targeting HDL from a mere increase in HDL concentration toward improving HDL function. This view is further supported by the recently published dal-OUT-COMES trial, documenting that just increasing HDL level may not reduce the risk of recurrent cardiovascular events.

To assess the relation between disease severity of patients with CHF and HDL function, we isolated HDL from healthy controls and CHF patients in NYHA class II and IIIb and determined its ability to stimulate eNOS activation and thereby NO production. To study the impact of ET on HDL function, HDL was additionally isolated from healthy individuals and NYHA-IIIb patients after an ET program, and its stimulating effect on eNOS phosphorylation and NO production was evaluated.

**Methods**

**Patient Population and Blood Sampling**

Sixteen healthy control subjects with normal systolic left ventricle function (>55%), without signs or symptoms of CHF as well as evidence of coronary artery disease during coronary angiography, were included in the study. The healthy control subjects performed an ET program as outlined below for 4 weeks. In addition, 8 patients with CHF (LVEF <40%) in NYHA class II and 16 CHF patients in NYHA class IIIb were recruited. Patients in NYHA-IIIb were assigned to a 12-week ET program as outlined below.

Blood was collected from all subjects when entering the study and when finishing the ET program (only healthy and NYHA-IIIb subjects). Serum was prepared by centrifugation (10 minutes at 3000 g) and analyzed in triplicate.

**Training Protocol**

NYHA-IIIb. The initial phase of the exercise program was supervised and performed in-hospital. During the first 3 weeks, patients exercised 3 to 4 times daily for 5 to 20 minutes on a bicycle ergometer adjusted to the workload at which 50% of maximum oxygen uptake (VO₂max) was reached. Before discharge from the hospital, symptom-limited spiroergometry was performed again to determine the training target heart rate for home-based training (defined as the heart rate reached at 60% of VO₂max). On discharge, patients were provided with bicycle ergometers for home-based ET. They were encouraged to exercise close to their target heart rate for 20 to 30 minutes daily for a period of 12 weeks and were expected to participate in a supervised group training session for 60 minutes every week consisting of walking, calisthenics, and noncompetitive ball games.

Healthy Controls. The complete ET program was supervised and performed in-hospital. The participants exercised at 65% to 75% of VO₂max during a period of 4 weeks (4 times 30 minutes per day, 5 days per week) on a bicycle ergometer.

The total exercise time or exercise volume was not different between both groups.

**Measurement of Endothelial Function**

Flow-mediated dilatation of the radial artery was measured using a high-resolution ultrasound scanning echo-tracking angiometer (NIUS 02; Asulab Research Laboratory, Neuchatell, Switzerland). For detailed description, see Online Data Supplement.

**Isolation of HDL**

HDL was isolated from serum by sequential density ultracentrifugation (d=1.006–1.21 g/mL) as recently described in detail. For detailed description, see Online Data Supplement.

**Cell Culture and Incubation With Isolated HDL**

Human aortic ECs (HAEC; Cell Systems Biotechnology, Troisdorf, Germany) were cultured in EGM-2 cell culture medium (Lonza, Walkersville, MD) until 80% to 90% confluence. Cells were incubated for 0, 5, 10, 15, 30, or 60 minutes with 50 µg/mL isolated HDL. To elucidate signaling pathways, cells were pretreated (1 hour before HDL stimulation) with specific inhibitors such as rapamycin (20 nM/L; Santa Cruz, Heidelberg, Germany) to inhibit PKC-β. Thereafter, cells were harvested with ice-cold lysis buffer (50 mM/L Tris-HCl; pH 7.4; 1% NP-40; 0.25% Na-deoxycholate; 150 mM/L NaCl; 1 mM/L EDTA; 0.1% Triton X-100; 0.2% SDS) containing protease inhibitor mix M (Serva, Heidelberg, Germany) as well as phosphatase inhibitor mix II (Serva). Protein concentration was determined using BSA as standard (BCA method; Pierce, Rockford, IL).

**Western Blot Analysis**

Ten micrograms of total protein were separated on a denaturing polyacrylamid gel and transferred to a PVDF membrane. To detect specific proteins, the following antibodies were applied: anti-eNOS (Santa Cruz), antiphospho-eNOS-Ser1177, antiphospho-eNOS-Thr595 (both BD Biosciences, Heidelberg, Germany), anti-PKCI-β, antiphospho-PKC-βII-Ser660, anti-p70S6K, antiphospho-p70S6K (all Santa Cruz). For the evaluation of HDL-induced phosphorylation of the respective protein, the maximal stimulation was used. All samples were analyzed in triplicate.

**Measurement of EC NO Production by ESR Spectroscopy**

HAECs were incubated with HDL (50 µg/mL) for 60 minutes. NO production in HAEC was measured by ESR spectroscopy using the spin-probe colloid Fe(DETC)₃. For detailed description, see Online Data Supplement.

**Measurement of Paraoxonase-1 Enzymatic Activity**

Paraoxonase (Pon) activity of HDL-associated paraoxonase-1 (PON1) was measured by spectrophotometry using paraoxon as substrate.

**Quantification of Protein-Bound Malondialdehyde in HDL**

Free and protein-bound MDA in HDL was detected by a commercially available lipid peroxidation assay kit (ALDetect; Enzo Life Sciences) as recommended by the manufacturer.

**Measurement of Plasma Oxidative Capacity**

Oxidative capacity was determined in plasma samples from all participants using a commercially available quantification kit (PerOx; KC5100; Immundinagnostik, Bensheim, Germany). Measurements were performed in duplicate.
NanoLC-MS/MS Analysis
The proteome of isolated HDL particles was investigated by shotgun LC-MS/MS analysis. For detailed description, see Online Data Supplement.

Statistical Analysis
SPSS version 16.0 (SPSS Inc, Chicago, IL) was used for all the analyses. Data are expressed as mean±SEM. Comparisons among groups were tested with ANOVA. When data were not normally distributed or the variance was not equal, the Kruskal–Wallis nonparametric test was used. A value of *P*<0.05 was considered statistically significant. All measurements were made by investigators blinded to the treatment group.

Results

**Patient Characteristics and Follow-up After ET**
The baseline values for all individuals included into the study are depicted in Online Table I. As expected, the healthy individuals significantly differed from patients with CHF with respect to left ventricle ejection fraction, maximal oxygen consumption, and medication. No difference was observed among the 3 groups comparing age, body mass index, arterial hypertension, and diabetes mellitus. None of the CHF patients were active smokers, whereas 3 healthy controls smoked at study beginning. On analyzing the lipid profile, a significantity between the groups were also evident when measuring the differences in HDL-mediated eNOS phosphorylation capacity between the groups were also evident when measuring the HDL-induced NO production of ECs.

Comparing the functional properties of HDL_{NYHA-IIIb} before and after an exercise program revealed a significant improvement in phosphorylation of eNOS at position Ser^{1177} (beginning: 1.7±0.2-fold versus unstimulated cells; end: 3.3±0.5-fold versus unstimulated cells; *P*<0.01; Figure 1A), a significant reduction in phosphorylating eNOS at position Thr^{495} (beginning: 3.3±1.0-fold versus unstimulated cells; end: 2.1±0.4-fold versus unstimulated cells; *P*<0.05).

**HDL-Mediated eNOS Phosphorylation and NO Production: Impact of ET**

In incubating HAECs with HDL_{healthy}, phosphorylation of eNOS at position Ser^{1177} was increased 4.7±0.4-fold versus unstimulated cells. This stimulation of eNOS-Ser^{1177} phosphorylation was lower with HDL_{NYHA-IIIb} (3.7±1.1-fold versus unstimulated cells; *P*<0.05 versus healthy controls), reaching significantly lower values with HDL_{NYHA-IIIb} compared with HDL_{healthy} (1.7±0.2-fold versus unstimulated cells; *P*<0.001 versus healthy controls; Figure 1A). Analyzing the eNOS phosphorylation at position Thr^{495}, HDL_{NYHA-IIIb} stimulated phosphorylation significantly more than by HDL_{healthy} (NYHA-IIIb: 3.3±0.4-fold versus unstimulated cells; NYHA-II: 2.6±0.5-fold versus unstimulated cells; healthy controls: 1.5±0.4-fold versus unstimulated cells; *P*<0.05 NYHA-IIIb versus healthy controls; Figure 1B). These differences in HDL-mediated eNOS phosphorylation capacity between the groups were also evident when measuring the HDL-induced NO production of ECs (Figure 2).

After 12 weeks of ET was evident. In the healthy controls, ET resulted in an increase in VO_{2max}, a decrease in LDL, and an increase in HDL. All other parameters were unchanged.

![Graph showing HDL-mediated eNOS phosphorylation and NO production](https://example.com/graph.png)

**Figure 1.** Human aortic endothelial cells were incubated with high-density lipoprotein (HDL; 50 µg/mL) isolated from healthy controls (healthy) at beginning (Beg.) and after performing an exercise training program (End), chronic heart failure (CHF) patients in NYHA class-II (NYHA-II), and CHF patients in NYHA class-III at beginning (Beg.) and after finishing an exercise training program (End), and phosphorylation of endothelial NO synthase (eNOS) at position Ser^{1177} (A) and Thr^{495} (B) was evaluated by Western blot analysis. For quantitative analysis, the x-fold increase in eNOS phosphorylation of HDL-incubated cells vs untreated cells was determined. Representative examples of the Western blots are depicted on top of the figure. Values are expressed as means±SEM.
versus unstimulated cells; \( P < 0.05 \); Figure 1B), and a significantly higher production of NO in ECs (beginning: -1.0±3.3% increase versus buffer-treated cells; \( P < 0.01 \); Figure 2). No significant impact of ET was observed in the healthy control group with respect to HDL-mediated eNOS-Ser\(^{1177}\) phosphorylation (beginning: 4.7±0.4-fold versus unstimulated cells; \( P = \text{NS} \), eNOS-Thr\(^{495}\) phosphorylation (beginning: 1.5±0.4-fold versus unstimulated cells; \( P = \text{NS} \), Figure 1A and 1B), and NO production (Figure 2).

**HDL-Mediated PKC-\(\beta\)-II Ser\(^{666}\) Phosphorylation**

Incubation of HAECs with HDL\(_{\text{healthy}}\) resulted in a 1.4±0.3-fold increase in PKC-\(\beta\)-II phosphorylation at position Ser\(^{666}\) as compared with unstimulated cells. The ability to stimulate PKC-\(\beta\)-II phosphorylation was increased with increasing severity of CHF (NYHA-II: 1.8±1.1-fold versus unstimulated cells; NYHA-IIIb: 2.2±0.7-fold versus unstimulated cells; \( P < 0.05 \) NYHA-IIIb versus healthy; Figure 3A). In patients with CHF NYHA class IIIb, performing an exercise program significantly reduced the ability of HDL\(_{\text{NYHA-IIIb}}\) to phosphorylate PKC-\(\beta\)-II (beginning: 2.2±0.7 versus end: 1.4±0.2-fold versus unstimulated cells; \( P < 0.05 \); Figure 3A). No impact of ET on HDL-induced phosphorylation of PKC-\(\beta\)-II was observed in healthy controls (beginning: 1.4±0.3 versus end: 1.6±0.2-fold versus unstimulated cells; \( P = \text{NS} \); Figure 3A).

To evaluate the importance of PKC-\(\beta\)-II activation for HDL-induced eNOS phosphorylation, the HDL\(_{\text{NYHA-IIIb}}\)-induced eNOS phosphorylation at Ser\(^{1177}\) and Thr\(^{495}\) was measured in the absence or presence of a specific PKC-\(\beta\)-II inhibitor (CG53353). As shown in Figure 3B, the inhibition of PKC-\(\beta\)-II prevented HDL-induced phosphorylation at eNOS-Thr\(^{495}\) without having any influence on eNOS-Ser\(^{1177}\) phosphorylation.

**HDL-Mediated p70S6K-Ser\(^{411}\) Phosphorylation**

Incubation of HAECs with HDL\(_{\text{healthy}}\) resulted in a 1.5±0.2-fold increase in p70S6K phosphorylation at position Ser\(^{411}\) as compared with unstimulated cells. This ability to stimulate p70S6K phosphorylation was increased with increasing severity of CHF (NYHA-II: 1.9±0.4-fold versus unstimulated cells; NYHA-IIIb: 3.1±0.7-fold versus unstimulated cells; \( P < 0.05 \) NYHA-IIIb versus healthy; Figure 4A). In patients with CHF NYHA class IIIb, performing an exercise program significantly reduced the ability of HDL\(_{\text{NYHA-IIIb}}\) to phosphorylate PKC-\(\beta\)-II (beginning: 3.1±0.7 versus end: 1.4±0.1-fold versus unstimulated cells; \( P < 0.01 \); Figure 4A). No impact of ET on HDL-induced phosphorylation of PKC-\(\beta\)-II was observed in healthy controls (beginning: 1.5±0.2 versus end: 1.7±0.2-fold versus unstimulated cells; \( P = \text{NS} \); Figure 4A).

Blocking PKC-\(\beta\)-II activation by CG53353 resulted in an inhibition of HDL\(_{\text{NYHA-IIIb}}\)-induced phosphorylation of p70S6K at Ser\(^{411}\) (Figure 4B). In addition, blocking p70S6K activation by rapamycin prevented HDL\(_{\text{NYHA-IIIb}}\)-induced phosphorylation of eNOS at Thr\(^{495}\) without influencing eNOS phosphorylation at Ser\(^{1177}\) (Figure 4C and 4D).

**MDA Bound to HDL**

HDL-bound MDA was significantly increased in HDL\(_{\text{NYHA-IIIb}}\) as compared with HDL\(_{\text{healthy}}\) (healthy: 0.39±0.09 nmol/mg HDL; NYHA-II: 0.64±0.09 nmol/mg HDL; NYHA-IIIb: 1.13±0.10 nmol/mg HDL; \( P < 0.001 \) healthy versus NYHA-IIIb; \( P = 0.05 \) NYHA-II versus NYHA-IIIb; Figure 5A). After finishing an ET program, the amount of HDL-bound MDA was significantly reduced by 27.4±6.1% in CHF-NYHA-IIIb, whereas no change was observed in healthy controls (Figure 5B).

**PON-1 Enzyme Activity and Total Lipid Peroxides**

Measuring the PON enzymatic activity of PON-1 associated with isolated HDL, a significant decrease in HDL\(_{\text{NYHA-IIIb}}\) was evident as compared with HDL\(_{\text{healthy}}\) (Figure 5C). Although exercise intervention triggered a further increase in PON-1 activity of HDL\(_{\text{healthy}}\), it did not result in an improvement of low PON-1 activity observed for HDL\(_{\text{NYHA-IIIb}}\) (Figure 5C).

Evaluating circulating total lipid peroxides as a measure for oxidative status, a significant higher level was obvious in the plasma of CHF NYHA-IIIb patients as compared with that of healthy controls (healthy: 151±41 versus NYHA-IIIb: 351±71 \( \mu \)mol/L; \( P < 0.05 \)). Furthermore, the ET intervention led to a significant reduction of total lipid peroxides (beginning NYHA-IIIb: 351±71 versus end: NYHA-IIIb: 210±54 \( \mu \)mol/L; \( P < 0.05 \); Figure 5D). No impact of ET on total lipid peroxides was evident in healthy controls (Figure 5D).

**Correlation Between HDL and Endothelial Function**

To investigate if the exercise-induced change in HDL function observed in CHF NYHA-IIIb patients has some influence on endothelial function, a correlation analysis between the change in HDL function and the observed change in endothelial function was performed. A significant correlation was evident between the change in HDL-induced eNOS-Ser\(^{1177}\) phosphorylation (\( r = 0.83; \ P < 0.0001 \); Figure 6A) or the absolute change in HDL-induced NO production (\( r = 0.78; \ P < 0.05 \); Figure 6B) and the change in endothelial function.
Proteome Analysis of HDL Particles

In LC-MS/MS analysis of HDL particles, 709 distinct peptides covering 134 distinct proteins were identified, applying a MASCOT cut-off ion score of >23 and a false discovery rate of <1%. We identified additional protein groups that have not been described in HDL particles before, such as β-Ala-His dipeptidase (CNDP1; Online Table II).

Differences in the proteomic profile of HDLs from healthy controls and CHF-NYHA-IIIb patients as well as effects of ET were visualized using a principal component analysis plot as indicator of variance in the data set (n=134 proteins;
Figure 4. Human aortic endothelial cells (HAECs) were incubated with high-density lipoprotein (HDL; 50 μg/mL) isolated from healthy controls (healthy) at beginning (Beg.) and after finishing an exercise training (ET) program (End), chronic heart failure (CHF) patients in NYHA class II (NYHA-II), and CHF patients in NYHA class III at beginning (Beg.) and after finishing an ET program (End), and phosphorylation of p70S6K at position Ser411 was evaluated by Western blot analysis (A). HAECs were incubated with HDL (50 μg/mL) isolated from CHF-NYHA-IIIb patients in the presence or absence of a PKC-β inhibitor (+/− CG53353; B) or the p70S6K inhibitor rapamycin (+/− rapamycin; C) and phosphorylation of p70S6K-Ser411 (B) or phosphorylation of eNOS-Thr495 (C) and eNOS-Thr417 (D) was evaluated. To compensate for protein loading differences, phosphorylation at the specific site was normalized to the nonphosphorylated form. For quantitative analysis, the x-fold increase in PKC-β phosphorylation of HDL-incubated cells vs untreated cells was determined. Representative examples of Western blots are depicted on top of the figure. Values are expressed as means±SEM.

Discussion

In recent years, it became evident that not only the quantity, but also the functional capacity of HDL is important for influencing the risk of cardiovascular disease. Therefore, strategies are being developed to increase HDL quantity and function. ET is an accepted intervention strategy in patients with systolic heart failure, and the molecular mechanisms behind the beneficial effect of ET have been described in part (for a detailed review, see Gielen...
et al.21). With respect to functional properties of HDL in patients with CHF and the impact of ET, several findings emerge from the present study. First, the functional capacity of HDL to increase NO production in ECs via modulation of eNOS phosphorylation is impaired in patients with CHF. This functional incompetence is gradually increasing with disease severity. Second, in CHF patients, the amount of MDA bound to HDL is significantly elevated and the activation of PKC-βII/p70S6K by HDL\textsubscript{NYHA-IIIb} is significantly increased as compared with HDL\textsubscript{healthy}. Third, ET of CHF patients in NYHA-IIIb for 12 weeks significantly restored the functional capacity of HDL as well as the amount of MDA bound to HDL and the HDL-mediated activation of PKC-βII/p70S6K. The ET-induced change in HDL function significantly correlated with an improved endothelial function. Fourth, proteome analysis of the HDL particles revealed a clear distinction between HDL isolated from healthy controls and CHF NYHA-IIIb patients. No significant impact of ET on the overall protein composition of HDL particles was evident.

These findings suggest that ET has a positive effect on HDL function with respect to NO generation, and this might be one pathway how ET improves endothelial function in patients with CHF.

**CHF and the Ability of HDL to Regulate NO Production**

Experimental and clinical studies have suggested that eNOS-derived NO is a crucial determinant of vascular homeostasis, and reduced bioavailability of NO plays an important role in the development and progression of atherosclerosis.22 Patients with CHF, irrespective of atherosclerotic etiology, are characterized by endothelial dysfunction as a result of increased oxidative stress and reduced NO bioavailability.23

The activity of eNOS is regulated by intracellular calcium concentration, protein concentration per se, or by phosphorylation at specific residues via activation of protein kinase B or protein kinase A (for a detailed review, see Kolluru et al.24). Besides the activation of eNOS by shear stress or agonists such as acetylcholine, bradykinin, or vascular endothelial growth factor, HDL also has the potency to activate eNOS by binding to the scavenger receptor-B1.25 In the present study, we described, for the first time, that the capacity of HDL isolated from CHF patients to phosphorylate eNOS and generate NO is significantly impaired. This reduced ability of HDL to stimulate eNOS activity and NO production even seems to be related to disease severity, because the most relevant impairment was seen in CHF patients in NYHA class IIIb followed by patients in NYHA-II as compared with healthy controls. This result is in good agreement with findings in other atherosclerotic disorders.13–15 This functional reduction of HDL is on top of a quantitative reduction of HDL, which was noted in patients described in the present study. With respect to functional properties of HDL in CHF patients in NYHA class II (NYHA-II; n=8), and CHF patients in NYHA class III (n=8; A) as well as the change after performing an exercise training program (B). PON1 paraoxonase activity was measured in HDL isolated from healthy controls (healthy) before (Beg.) and after an exercise training (ET) program (End), CHF patients in NYHA class II (NYHA-II), and CHF patients in NYHA class III at beginning (Beg.) and after finishing an ET program (End; C). In addition, the total amount of lipid peroxides as a measure for oxidative load was quantified in the plasma of all study participants (D). Values are expressed as mean±SEM.

**Figure 5.** Quantification of protein-bound malondialdehyde (MDA) in high-density lipoprotein (HDL) isolated from healthy controls (healthy; n=8), chronic heart failure (CHF) patients in NYHA class II (NYHA-II; n=8), and CHF patients in NYHA class III (n=8; A) as well as the change after performing an exercise training program (B). PON1 paraoxonase activity was measured in HDL isolated from healthy controls (healthy) before (Beg.) and after an exercise training (ET) program (End), CHF patients in NYHA class II (NYHA-II), and CHF patients in NYHA class III at beginning (Beg.) and after finishing an ET program (End; C). In addition, the total amount of lipid peroxides as a measure for oxidative load was quantified in the plasma of all study participants (D). Values are expressed as mean±SEM.

**Figure 6.** Correlation analysis between change in eNOS-Ser\textsuperscript{1177} phosphorylation (x-fold begin vs. 3 month) (n=16; A) and between change in NO production and change in endothelial function (n=8; B) for NYHA-IIIb patients of the training group.
Effect of ET on HDL Function

ET has been proven to correct endothelial dysfunction partially in a variety of diseases (for a review, see Gielen et al). Restoration of NO bioavailability due to an increase in eNOS expression, a protein kinase B–mediated phosphorylation of eNOS at position Ser1177 and Thr495, of note, this activation of the PKC–βII pathway was significantly reduced in CHF patients after an ET program. A central question based on this observation is: what discriminates HDL isolated from healthy controls and patients with CHF in NYHA-IIIb in its ability to activate PKC–βII/p70S6K? At least 2 possibilities should be discussed: first, a change in the overall protein composition of the HDL particle; and second, a post-translational modification of the HDL particle. With respect to protein composition, a clear difference was evident between healthy controls and CHF–NYHA-IIIb patients. However, ET did not result in a drastic modification of the HDL proteome despite an improved HDL-mediated NO production. Second, another potential mechanism would be the modification of apolipoprotein A1 by reactive intermediates. Indeed, impaired HDL function after modification by MDA or myristic acid could be documented. For example, MDA modification of HDL decreased cholesterol efflux from cultured human fibroblasts or rat liver ECs. Notably, in the present study, we observed that a significant higher level of MDA is bound to HDL NYHA-IIIb in comparison with HDL healthy, and that a training intervention significantly reduced the MDA amount bound to HDL NYHA-IIIb. Supported by in vitro studies, it is reasonable to assume that the amount of MDA bound to HDL regulates the activation of PKC–βII (higher activation of PKC–βII in case more MDA is bound to HDL) and, finally, the activation of eNOS and NO generation. Because the modulation of HDL by MDA seems to play such a central role, the question that arises is: which factors are regulating the amount of MDA bound to HDL, and how are they influenced by ET? Pon, an enzyme associated with HDL, protects lipoproteins (HDL and LDL) from oxidative modification, and in clinical studies, a negative correlation between Pon activity and cardiovascular risk was confirmed. In the present study, Pon activity of HDL NYHA-IIIb was significantly decreased when compared with HDL healthy. However, ET had no effect on Pon activity. This is in accordance with a study performed in obese men, showing improved HDL inflammatory/anti-inflammatory properties after a lifestyle
intervention without changing Pon activity. Therefore, at the moment it remains unclear which mechanism beside Pon activity may be responsible for the ET-mediated reduction in MDA bound to HDL. Of note, an influence of ET on the total oxidative capacity, measured as lipid peroxides in the patient plasma, was evident. One may speculate that ET reduces the oxidative load and thereby reduces the amount of MDA bound to HDL.

Study Limitations

Some limitations of the present study should be mentioned. First, only 16 NYHA-III patients or healthy individuals and 8 NYHA-II patients were included into the study. Nevertheless, the primary goal of this study was to investigate if HDL function in patients with CHF was altered and if this could be reversed by ET. Even with such a small number of individuals in each group, a significant impaired HDL function in NYHA-IIb was evident, which could be improved by ET. Unfortunately, no data are available on the impact of ET in CHF patients in NYHA-class II, and we cannot rule out the impact of differences in medication background.

Second, it remains unclear which mechanism is responsible for the exercise-induced reduction of MDA bound to HDL. According to the data presented, the activity of paraoxogenase is not altered by ET and, therefore, can be excluded. However, we could observe an exercise-induced reduction in lipid peroxidation, which is a general measure for oxidative load. So far, it remains unclear which enzymatic system is responsible for the observed reduction in oxidative stress.

Third, currently we do not know if the alterations in HDL proteome observed between HDL_{healthy} and HDL_{NYHA-IIb} have any causal relevance for HDL-mediated eNOS phosphorylation. To answer these questions, extensive studies using site-directed mutagenesis are warranted. Because the proteome seems not to be influenced by ET, one may speculate that the HDL particle composition may not be as critical for HDL-mediated eNOS regulation as the MDA bound to the HDL particle.

Fourth, the results presented are obtained in cell culture experiments using HDL isolated from frozen serum of different patient cohorts. Therefore, it has to be answered if this modulation of HDL also has an impact on endothelial function in vivo. At least the correlation detected between improved endothelial function induced by ET measured in vivo and HDL-associated change in eNOS phosphorylation and NO generation measured in vitro implies that this is an important mechanism for the regulation of endothelial function in vivo. With respect to serum storage at −80°C and functionality, no data with respect to HDL-mediated eNOS phosphorylation are available. Nevertheless, in a recent study investigating the HDL-mediated cholesterol efflux, no difference was detected between HDL isolated from fresh serum and serum stored for up to 2 years.

Conclusions

In summary, the reduced ability of HDL to stimulate endothelial NO production via activation of eNOS in patients with advanced CHF suggests a loss of this functional property of HDL. An ET program in this patient cohort seems to correct this dysfunction partially. This may be one possible explanation, beside several others, for the beneficial effect of ET on...
endothelial function. The exercise-mediated effect on HDL function seems to be mediated via a reduction in MDA bound to HDL, leading finally to a lower activation of PKC-βII resulting in a higher activation of eNOS and a higher synthesis of NO (Figure 8).

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Disclosures

None.

References

What Is Known?
- Endothelial function is significantly impaired in patients with chronic heart failure (CHF), partly because of a reduction in NO bioavailability.
- Exercise training (ET) can restore endothelial function in patients with CHF.
- High-density lipoprotein (HDL)-mediated NO production in endothelial cells is impaired in patients with diabetes mellitus and cardiovascular diseases.

What New Information Does This Article Contribute?
- HDL-mediated NO production via activation of endothelial NO synthase (eNOS) is significantly impaired in patients with heart failure.
- Reduction in HDL-mediated eNOS activation correlates with the severity of heart failure.
- Aerobic ET over a 12-week period partially restores HDL-mediated NO production in the ECs in patients with heart failure.
- This improvement in endothelial function by ET is probably because of a reduction in HDL-bound malondialdehyde (MDA) resulting in a lesser inhibition of eNOS.

The study was designed to investigate the impact of aerobic continuous ET on HDL-mediated regulation of eNOS activation and NO production in patients with CHF. Isolated HDL from CHF patients exhibited a lower capacity to activate eNOS and produce NO, as compared with healthy controls. Impaired NO production correlated with the severity of heart failure. Twelve weeks of ergometer training partially restored impaired HDL function in activating eNOS in CHF patients in NYHA class IIIb. Partial recovery of HDL-mediated eNOS activation correlated with an improvement in endothelial function. No effect of ET was observed in healthy controls. Exercise-mediated improvement of HDL function was associated with a reduction of HDL-bound MDA level and a lower activation of PKC-βII. The results suggest that ET is an important modulator of HDL function in activating eNOS and generating NO in patients with chronic heart failure.
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Measurement of endothelial function

A high-resolution echo-tracking angiometer (NIUS 02, Asulab Research laboratory, Neuchatel, Switzerland) was used for non-invasive measurement of radial artery internal diameter. FMD measurements were performed before exercise testing. After a rest period of at least 20 minutes and under standardized conditions (quite, temperature-controlled room, fasting condition) baseline diameter was determined. A 10 MHz transducer was positioned perpendicular to the radial artery about 5 cm proximal to the wrist, without direct skin contact. During measurements the patient was in supine position with the forearm resting on a special support device to avoid unintentional motions. For determination of FMD, the brachial artery was occluded by inflating a blood pressure cuff to 50 mmHg above the systolic blood pressure for 5 minutes. The radial artery internal diameter was continuously recorded at least 180 seconds after cuff release, and the maximal diameter was recorded and related to baseline diameter to determine FMD. Only one experienced investigator (S.E.), who was blinded to patient identity, group assignment, and intervention status performed FMD measurements.

Isolation of HDL

Serum aliquots (1-2 ml) were mixed with KBr solution (d=1.006) and centrifuged for 24h in an ultracentrifuge (T865 rotor, 50’000 rpm; 4°C, 10.4 ml tubes). After the centrifugation, the lower half of the centrifuge tube was transferred to a new centrifuge tube containing 0.941 g solid KBr. The tube was filled with KBr solution (d=1.006) and centrifuged for 24h (T865 rotor, 50’000 rpm; 4°C). Once again, the lower half of the tube was transferred to a new tube containing 2.251 g of KBr, filled with KBr (d=1.063) and centrifuged for 48h using an ultracentrifuge (T865 rotor, 50’000 rpm; 4°C). After this step, the upper part was transferred to a new tube, and after filling the tube with KBr (d=1.21) another centrifugation step in the ultracentrifuge for 24h was performed (T865 rotor, 50’000 rpm; 4°C). The last step was repeated again, and after this centrifugation step the upper part was collected and concentrated using an Ultracel 30K (Millipore, Darmstadt, Germany). The concentrate was washed several time using Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.25 mM CaCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 11 mM glucose, pH 7.2) and stored at 4°C after sterile filtration until used in the cell culture experiments. Quality of isolated HDL was evaluated by polyacrylamid gel electrophoresis followed by coomassie blue staining.

Measurement of endothelial cell NO production by ESR spectroscopy

The effects of HDL (50 µg/ml; 60 min, 37°C) on endothelial NO production (HAECs; passage 4-7; Cambrex Bio Science) was examined by electron spin resonance (ESR) spectroscopy using the spin-probe colloid Fe(DETC)₂ (Noxygen), as described and validated previously. ESR spectra of samples frozen in liquid nitrogen were recorded on a Bruker e-scan spectrometer (Bruker BioSpin) with the following instrumental settings: center field (B0) 3455 G, sweep width 80 G, microwave power 39.72 db, amplitude modulation 10.34 G, sweep time 10.49 sec, number of scans 10.
Measurement of PON1 activity

Paraoxonase activity of HDL-associated paraoxonase was measured by spectrophotometry in a 96-well plate format using paraoxon (Sigma-Aldrich, Taufkirchen, Germany) as substrates, as previously described. For paraoxonase activity assays, HDL was diluted in reaction mixture containing 10 mM Tris hydrochloride (pH 8.0), 1 M sodium chloride and 2 mM calcium chloride. The reaction was initiated by addition of 1.5 mM paraoxon and the increase in absorbance at 405 nm due to the generation of para-nitrophenol was recorded. An extinction coefficient of 17,000 M⁻¹ cm⁻¹ (at 24°C) was used for calculating units of paraoxonase activity, which are expressed as the amount of paranitrophenol produced in nanomoles per minute per milligram of HDL. Paraoxonase activity assays for each sample were performed in triplicates and average measurements of enzyme activity were calculated for each sample. Each 96-well plate included blank samples to monitor spontaneous hydrolysis of substrates.

NanoLC-MS/MS analysis

The proteome of isolated HDL particles from 7 NYHA-IIIb patients and 8 healthy controls before and after an ET program were investigated by shotgun LC-MS/MS. In total, 4 µg of protein lysates were reduced (2.5 mM DTT for 1 h at 60°C) and alkylated (10 mM iodacetamide for 30 min at 37°C). Proteolysis was performed overnight using trypsin (Promega, Madison, WI) with a ratio of 1:25 at 37°C. The tryptic digestion was stopped by adding acetic acid at the final concentration of 1%, followed by desalting and purification using ZipTip-µC18 tips (Millipore, Billerica, MA).

Proteolytically cleaved peptides (500 ng) were, prior to mass spectrometric analyses, enriched on a 2 cm Acclaim PepMap100-precolumn (C18 2 µm, 100 Å) and then separated by reverse phase nano HPLC on a 15 cm Acclaim PepMap RSLC-column (C18 2 µm, 100 Å) using a Dionex UltiMate 3000 RSLCnano system (Thermo Scientific, Waltham, MA) at a constant flow rate of 300 nL/min. Separation was achieved using a linear gradient of 60 min (2%-25%) with 0.1% acetic acid, 2% acetonitrile in water (solvent A) and 0.1% acetic acid in 100% acetonitrile (solvent B). Separated peptides were monitored using a QExactive mass spectrometer (Thermo Scientific) equipped with a TriVersa NanoMate ion source (Advion BioSystems, Ithaca, NY). The MS-instrument was operated in data-dependent acquisition (DDA) mode. MS settings were as follow: survey full-scan spectra were acquired with a resolution R = 70,000, automated gain control (AGC) target was set to 3e6 ions, the maximum injection time was set to 250 ms. MS/MS scan events were repeated for top 10 peaks using the higher energy dissociation mode (HCD) at normalized collision induced energy of 27.5%, underfill ratio (5%) with an intensity threshold of 8.3e4 ions was selected. Already targeted ions for MS/MS were dynamically excluded for 30 s with monoisotopic precursor selection enabled. Raw data from the Q Exactive instrument was processed using the Refiner MS 7.5 and Analyst 7.5 module (Genedata, Basel, Switzerland). Refiner MS performed peak detection, noise analysis, peak integration, isotope grouping and retention time alignment of the LC-MS/MS data. Generated peak lists were searched against a human FASTA-formatted database containing 20,268 unique entries (human_uniprot_swatchprot_2011_10.fasta) using an in-house Mascot server v2.3.2 (Matrix Science, London, GB). Database searches were performed with carbamidomethyl on cysteine as fixed modification and oxidation on methionine as variable modification. Enzyme specificity was selected to trypsin with up to two missed cleavages allowed using 10 ppm peptide ion tolerance and 20 mmu MS/MS tolerance. Only ranked one peptide hits and a Mascot ion score
>23 were considered as identified. After peak annotation, the data were further processed in Analyst 7.5, where statistical data evaluation was performed using univariate and multivariate methods. Paired sample T-Test and parametric T-test were used for group comparison. A value of p<0.05 was considered as statistically significant. A Principal Components analysis (PCA) was applied as classical means of dimensionality reduction and visualization of multivariate data. Proteins were functionally assigned to canonical pathways using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA).

References


Supplementary Table I: Clinical characteristics and cardiovascular medication

<table>
<thead>
<tr>
<th></th>
<th>Healthy Begin (n=16)</th>
<th>Healthy End (n=16)</th>
<th>CHF NYHA-II (n=8)</th>
<th>CHF NYHA-IIIb Begin (n=16)</th>
<th>CHF NYHA-IIIb End (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age [years]</td>
<td>68±2</td>
<td>68±2</td>
<td>54±4</td>
<td>63±2</td>
<td>63±2</td>
</tr>
<tr>
<td>Body mass index [kg/cm²]</td>
<td>27.3±1.0</td>
<td>27.6±0.9</td>
<td>30.6±1.5</td>
<td>28.7±0.7</td>
<td>28.3±0.8</td>
</tr>
<tr>
<td>Arterial hypertension [n]</td>
<td>14 (87.5%)</td>
<td>14 (87.5%)</td>
<td>3 (37.5%)</td>
<td>16 (100%)</td>
<td>16 (100%)</td>
</tr>
<tr>
<td>Diabetes mellitus [n]</td>
<td>2 (12.5%)</td>
<td>2 (12.5%)</td>
<td>1 (12.5%)</td>
<td>3 (18.8%)</td>
<td>3 (18.0%)</td>
</tr>
<tr>
<td>Active smoking [n]</td>
<td>3 (18.7%)</td>
<td>1 (6.3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>LV ejection fraction [%]</td>
<td>62±2</td>
<td>60±3</td>
<td>26±2 **</td>
<td>26±1 **</td>
<td>32±2 †</td>
</tr>
<tr>
<td>Peak VO₂ [ml/kg*min]</td>
<td>21.9±1.0</td>
<td>24.7±1.2 §</td>
<td>17.6±1.1 *</td>
<td>14.9±0.6 **</td>
<td>16.8±0.9 †</td>
</tr>
</tbody>
</table>

Etiology of heart failure

<p>| | | | | | |</p>
<table>
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</thead>
<tbody>
<tr>
<td>Ischemic heart disease [n]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilative cardiomyopathy [n]</td>
<td></td>
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</tbody>
</table>

Cardiovascular medication

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</thead>
<tbody>
<tr>
<td>Beta blocker [n]</td>
<td>12 (75%)</td>
<td>12 (75%)</td>
<td>5 (62.5%)</td>
<td>15 (93.4%)</td>
<td>15 (93.4%)</td>
</tr>
<tr>
<td>ACE inhibitor or ATII blocker [n]</td>
<td>6 (37.5%)</td>
<td>6 (37.5%)</td>
<td>8 (100%) *</td>
<td>16 (100%) *</td>
<td>16 (100%) *</td>
</tr>
<tr>
<td>Aldosterone antagonist [n]</td>
<td>0 (0%)</td>
<td>4 (25.0%)</td>
<td>3 (37.5%) *</td>
<td>16 (100%) *</td>
<td>16 (100%) *</td>
</tr>
</tbody>
</table>

Lipid profile

<p>| | | | | | |</p>
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol [mmol/L]</td>
<td>5.5±0.4</td>
<td>4.7±0.3</td>
<td>6.3±0.3</td>
<td>5.3±0.3</td>
<td>4.7±0.3</td>
</tr>
<tr>
<td>LDL cholesterol [mmol/L]</td>
<td>3.2±0.2</td>
<td>2.7±0.2 ^</td>
<td>4.3±0.4</td>
<td>3.3±0.3</td>
<td>2.8±0.2 †</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------</td>
<td>-----------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>HDL cholesterol [mmol/L]</td>
<td>1.4±0.2</td>
<td>1.5±0.2 ^</td>
<td>1.4±0.1</td>
<td>1.2±0.1 *§</td>
<td>1.2±0.1</td>
</tr>
</tbody>
</table>

Data are mean±SEM. Abbreviations: LV: left ventricular; peak VO2: peak oxygen consumption; CHF: chronic heart failure; ACE inhibitor: angiotensin converting enzyme inhibitor; ATII blocker: angiotensin II subtype I receptor blocker; LDL: low density lipoprotein; HDL: high density lipoprotein. * p<0.05 vs. healthy; ** p<0.001 vs. healthy; § p<0.05 vs. CHF-NYHA-II; † p<0.05 vs. CHF-NYHA-IIIb Beg., & p<0.05 vs. Healthy Beg.
**Supplementary Table II: Proteins identified by label free shotgun LC-MS/MS analysis**

<table>
<thead>
<tr>
<th>Protein name</th>
<th>UNI Prot Accession Numbers</th>
<th>Description</th>
<th>number of identified peptides</th>
<th>control vs. NYHA baseline</th>
<th>control vs. NYHA post</th>
<th>control pre vs. post</th>
<th>NYHA pre vs. post</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1AT</td>
<td>P01009 Alpha-1-antitrypsin</td>
<td></td>
<td>22</td>
<td>0.019 (1.77)</td>
<td>0.003 (2.09)</td>
<td>0.087</td>
<td>0.792</td>
</tr>
<tr>
<td>CO3</td>
<td>P01024 Complement C3</td>
<td></td>
<td>17</td>
<td>0.394</td>
<td>0.018 (-0.72)</td>
<td>0.843</td>
<td>0.083</td>
</tr>
<tr>
<td>APOM</td>
<td>O95445 Apolipoprotein M</td>
<td></td>
<td>9</td>
<td>0.01 (0.84)</td>
<td>0.006 (0.76)</td>
<td>0.435</td>
<td>0.846</td>
</tr>
<tr>
<td>APOC3</td>
<td>P02656 Apolipoprotein C-III</td>
<td></td>
<td>7</td>
<td>0.001 (-1.45)</td>
<td>0.001 (-1.1)</td>
<td>0.137</td>
<td>0.432</td>
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<tr>
<td>APOL1</td>
<td>O14791 Apolipoprotein L1</td>
<td></td>
<td>7</td>
<td>0.038 (-0.91)</td>
<td>0.111</td>
<td>0.245</td>
<td>0.87</td>
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<tr>
<td>APOC4</td>
<td>P55056 Apolipoprotein C-IV</td>
<td></td>
<td>5</td>
<td>0.001 (-1.62)</td>
<td>0.001 (-1.7)</td>
<td>0.644</td>
<td>0.588</td>
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<tr>
<td>SAA</td>
<td>P02735 Serum amyloid A protein</td>
<td></td>
<td>5</td>
<td>0.759</td>
<td>0.007 (-2.3)</td>
<td>0.094</td>
<td>0.287</td>
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<tr>
<td>CNDP1</td>
<td>Q96KN2* Beta-Ala-His dipeptidase</td>
<td></td>
<td>4</td>
<td>0.606</td>
<td>0.028 (0.75)</td>
<td>0.085</td>
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<tr>
<td>CO5</td>
<td>P01031 Complement C5</td>
<td></td>
<td>4</td>
<td>0.033 (-0.85)</td>
<td>0.109</td>
<td>0.841</td>
<td>0.538</td>
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<tr>
<td>APOC2</td>
<td>P02655 Apolipoprotein C-II</td>
<td></td>
<td>3</td>
<td>0.003 (-0.89)</td>
<td>0.005 (-0.84)</td>
<td>0.004 (0.19)</td>
<td>0.496</td>
</tr>
<tr>
<td>HPT</td>
<td>P00738 Haptoglobin</td>
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<td>2</td>
<td>0.017 (-1.87)</td>
<td>0.747</td>
<td>0.135</td>
<td>0.304</td>
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<tr>
<td>PCSK9</td>
<td>Q8NB07 Proprotein convertase subtilisin/kexin type 9</td>
<td></td>
<td>2</td>
<td>0.072</td>
<td>0.002 (-1.12)</td>
<td>0.63</td>
<td>0.397</td>
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<tr>
<td>THR8</td>
<td>P00734 Prothrombin</td>
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<td>2</td>
<td>0.003 (4.97)</td>
<td>0.001 (4.81)</td>
<td>0.228</td>
<td>0.344</td>
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<tr>
<td>ABC28</td>
<td>Q90428 ATP-binding cassette sub-family C member 8</td>
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<td>1</td>
<td>0.08</td>
<td>0.014 (2.3)</td>
<td>0.532</td>
<td>0.683</td>
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<tr>
<td>BBP1</td>
<td>Q8TDL5 BPI fold-containing family B member 1</td>
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<td>0.089</td>
<td>0.023 (-0.34)</td>
<td>0.296</td>
<td>0.239</td>
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<tr>
<td>DHX8</td>
<td>Q14562* ATP-dependent RNA helicase DHX8</td>
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<td>0.017 (2.4)</td>
<td>0.003 (2.89)</td>
<td>0.323</td>
<td>0.549</td>
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<tr>
<td>HRG</td>
<td>P04196 Histidine-rich glycoprotein</td>
<td></td>
<td>1</td>
<td>0.038 (2.11)</td>
<td>0.002 (2.37)</td>
<td>0.202</td>
<td>0.81</td>
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<tr>
<td>LBP</td>
<td>P18428 Lipopolysaccharide-binding protein</td>
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<td>1</td>
<td>0.037 (-1.01)</td>
<td>0.002 (-1.52)</td>
<td>0.697</td>
<td>0.207</td>
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<tr>
<td>SLIT1</td>
<td>Q75093* Slit homolog 1 protein</td>
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<td>1</td>
<td>0.256</td>
<td>0.042 (-0.83)</td>
<td>0.280</td>
<td>0.325</td>
</tr>
</tbody>
</table>

* novel identified proteins; group-wise comparison based on independent or dependent (paired) student T-Test. Data were represented by p-Value (effect size)