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Endothelial-vasoprotective effects of high-density lipoprotein are impaired in patients with type 2 diabetes mellitus but are improved after extended-release niacin therapy

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Abstract: **BACKGROUND:** High-density lipoprotein (HDL)-raising therapies are currently under intense evaluation, but the effects of HDL may be highly heterogeneous. We therefore compared the endothelial effects of HDL from healthy subjects and from patients with type 2 diabetes mellitus and low HDL (meeting the criteria for metabolic syndrome), who are frequently considered for HDL-raising therapies. Moreover, in diabetic patients, we examined the impact of extended-release (ER) niacin therapy on the endothelial effects of HDL. **METHODS AND RESULTS:** HDL was isolated from healthy subjects (n=10) and patients with type 2 diabetes (n=33) by sequential ultracentrifugation. Effects of HDL on endothelial nitric oxide and superoxide production were characterized by electron spin resonance spectroscopy analysis. Effects of HDL on endothelium-dependent vasodilation and early endothelial progenitor cell-mediated endothelial repair were examined. Patients with diabetes were randomized to a 3-month therapy with ER niacin (1500 mg/d) or placebo, and endothelial effects of HDL were characterized. HDL from healthy subjects stimulated endothelial nitric oxide production, reduced endothelial oxidant stress, and improved endothelium-dependent vasodilation and early endothelial progenitor cell-mediated endothelial repair. In contrast, these beneficial endothelial effects of HDL were not observed in HDL from diabetic patients, which suggests markedly impaired endothelial-protective properties of HDL. ER niacin therapy improved the capacity of HDL to stimulate endothelial nitric oxide, to reduce superoxide production, and to promote endothelial progenitor cell-mediated endothelial repair. Further measurements suggested increased lipid oxidation of HDL in diabetic patients, and a reduction after ER niacin therapy. **CONCLUSIONS:** HDL from patients with type 2 diabetes mellitus and metabolic syndrome has substantially impaired endothelial-protective effects compared with HDL from healthy subjects. ER niacin therapy not only increases HDL plasma levels but markedly improves endothelial-protective functions of HDL in these patients, which is potentially more important. **CLINICAL TRIAL REGISTRATION:** clinicaltrials.gov. Identifier: NCT00346970.

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**Endothelial-vasoprotective effects of HDL are impaired in patients with type-2 diabetes,
but are improved after extended-release niacin therapy**

Sorrentino – Endothelial effects of HDL in diabetes

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Abstract

Background: High-density-lipoprotein (HDL)-raising therapies are currently intensely evaluated. However, effects of HDL may be highly heterogenous. We therefore compared endothelial effects of HDL from healthy subjects (HS) and type-2 diabetics with low HDL (meeting criteria for metabolic syndrome), that are frequently considered for HDL-raising therapies. Moreover, in diabetics we examined the impact of extended-release(ER)-niacin therapy on endothelial effects of HDL.

Methods and Results: HDL was isolated from HS (n=10) and type-2 diabetics (n=33) by sequential ultracentrifugation. Effects of HDL on endothelial nitric oxide (NO) and superoxide production were characterized by electron-spin-resonance (ESR) spectroscopy analysis. Effects of HDL on endothelium-dependent vasodilation and early endothelial progenitor cell (EPC)-mediated endothelial repair were examined. Diabetics were randomized to a 3-month therapy with ER-niacin (1500 mg/d) or placebo, and endothelial effects of HDL were characterized.

HDL from HS stimulated endothelial NO production, reduced endothelial oxidant stress and improved endothelium-dependent vasodilation and early EPC-mediated endothelial repair. In contrast, these beneficial endothelial effects of HDL were not observed with HDL from diabetics, suggesting markedly impaired endothelial-protective properties of HDL. ER-niacin therapy improved the capacity of HDL to stimulate endothelial NO, to reduce superoxide production and to promote EPC-mediated endothelial repair. Further measurements suggested increased lipid oxidation of HDL in diabetics, and a reduction after ER-niacin therapy.

Conclusions: HDL from type-2 diabetics with metabolic-syndrome has substantially impaired endothelial-protective effects, as compared to HDL from healthy subjects. ER-

niacin therapy does not only increase HDL plasma-levels, but potentially more important, markedly improves endothelial-protective functions of HDL in these patients.

Key words:

High density lipoprotein (HDL); Endothelium; Nitric oxide; Niacin, Diabetes

Clinical Trial Registration Information-URL:

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Introduction

Reduced levels of HDL are a major risk factor for coronary disease,^{1, 2} and are predictive of cardiovascular events in patients treated with statins and low LDL cholesterol levels.³ Numerous recent studies have suggested that HDL exerts direct endothelial-protective effects, i.e. stimulates endothelial cell production of nitric oxide (NO)^{4, 5} and endothelium-dependent vasomotion,^{4,7} exerts antioxidant effects⁸ and promotes endothelial progenitor cell-mediated endothelial repair.^{9, 10} Notably, however, these studies have been performed using either HDL isolated from healthy subjects or reconstituted HDL. Given that HDL-raising strategies are currently intensely examined as a potential novel therapeutic approach to reduce cardiovascular events¹¹, it is critical to further characterize direct endothelial effects of HDL isolated from patients that are strongly considered for HDL-raising therapies.

Inhibition of the cholesteryl ester transfer protein (CETP) by torcetrapib resulted in a marked increase of HDL levels, however, was associated with an increased risk of mortality and morbidity of unknown mechanism in the recent ILLUMINATE trial.¹² In addition, no beneficial effects on carotid and coronary atherosclerosis progression were observed after torcetrapib therapy.¹²⁻¹⁴ This may, at least in part, be related to inherent adverse off-target effects of torcetrapib, i.e. an increased blood pressure, that is not observed with anacetrapib, another potent CETP-inhibitor.¹⁵ Conceivably, however, plasma levels of HDL may not represent a reliable surrogate endpoint to predict vasoprotective effects of HDL-targeted therapies.

Of note, it has been observed that the effects of HDL can be highly heterogenous. The ability of HDL to promote cholesterol efflux from macrophages by the ATP-binding cassette transporter A-1 (ABCA-1) pathway has been suggested to be variable and to be reduced by modification of HDL by pathophysiological concentrations of myeloperoxidase.¹⁶

Moreover, HDL isolated from patients with coronary disease exhibited a pro-inflammatory rather than an anti-inflammatory effect, that was partially attenuated in patients that were on statin therapy.¹⁷ In addition, the ability of HDL to counteract the inhibitory effect of oxidized LDL on vascular relaxation was reduced in type-2 diabetic patients.¹⁸

A careful understanding of the effects of HDL from diabetic patients with reduced HDL levels, that are frequently considered for HDL-boosting interventions, as compared to HDL from healthy subjects on endothelial cell nitric oxide and superoxide production, NADPH oxidase activity and endothelial progenitor cell-mediated endothelial repair is therefore required. More importantly, investigations assessing the effect of HDL-raising interventions on endothelial properties of HDL are urgently needed and may represent an attractive means to test the potential of such therapeutic approaches; i.e. it is conceivable that the increase in HDL confers cardiovascular protection only if pharmacologic interventions are associated with improved vasoprotective properties of HDL.

The present study therefore compared the endothelial effects of HDL isolated from patients with type-2 diabetes (meeting criteria for metabolic syndrome) and HDL isolated from healthy subjects. In particular, the effects of HDL on endothelial cell nitric oxide and superoxide production, NAD(P)H oxidase activity and early endothelial progenitor cell-mediated endothelial repair capacity were examined. Moreover, a randomised, controlled clinical study was performed to assess the effect of extended-release niacin therapy on these endothelial effects of HDL in patients with type-2 diabetes. Furthermore, potential mechanisms leading to changes in the endothelial effects of HDL were examined.

Methods

Patient Characteristics and Study Design. Written informed consent was obtained from all participants and the study protocol was approved by the local ethics committee. HDL and

EPCs were isolated from peripheral blood obtained from healthy subjects and type-2 diabetic patients. Patients with type-2 diabetes with reduced HDL cholesterol levels (<40 mg/dl in men; <50 mg/dl in women), meeting the criteria for the metabolic syndrome (as defined by the AHA and National Heart, Lung, and Blood Institute Scientific Statement¹⁹ and the International Diabetes Federation Metabolic Syndrome World-wide Definition²⁰) that were on statin therapy for at least three weeks or healthy subjects without cardiovascular risk factors or disease and without medication were included in the study. Endothelium-dependent vasodilation was examined by high-resolution ultrasound as described below. Characteristics of the study participants are shown in Table 1.

Diabetic patients ($n=33$) were then randomized (1:1) to receive a 3-month treatment with extended-release (ER)-niacin (Niaspan) or matching placebo. HDL was isolated after ER-niacin or placebo therapy and endothelium-dependent vasodilation was determined. ER-niacin was started with 500 mg/d, and the dosage was increased every month to achieve 1000 and 1500 mg/d, respectively. Placebo was started with one tablet/d, and the dosage was increased every month to achieve 2 and 3 tablets/d, respectively. The dose and timing of ER-niacin therapy were based on previous observations²¹ to achieve a significant increase of HDL plasma levels. One patient in the placebo group and two patients in the ER-niacin group discontinued therapy. There was no change in the medication of diabetic patients during the treatment period. Characteristics of the patients in both treatment groups are shown in Table 2.

Isolation of High-Density Lipoprotein. HDL was isolated from diabetic patients ($n=33$) and healthy subjects ($n=10$) from fresh, fasting plasma by ultracentrifugation ($d = 1.063-1.21$ g/ml) as described previously.^{4, 22} Protein, cholesterol and triglyceride content of HDL were

measured after isolation and are shown in Table 1 and 2. The concentrations of HDL used in the present study were based on protein content of HDL.

Effect of HDL on Endothelial Cell Nitric Oxide Production. HDL was administered to cultured human aortic endothelial cells (HAECs) and the effect on endothelial NO production was examined by electron spin resonance (ESR) spectroscopy analysis using the spin-trap colloid Fe(DETC)₂ as described in detail previously.^{23,24} In brief, HAECs were resuspended in 250µl of Krebs-Hepes buffer (37°C). 250µl of colloid Fe(DETC)₂ (final concentration 285µM) was added to each sample and incubated at 37°C for 60 min. The ESR spectra were recorded using a MiniScope ESR spectrometer (Magnettech). ESR instrumental settings were as follows: center-field (B₀) 3280G, sweep 198G, microwave power 4db, amplitude modulation 8G, 4096 points resolution, sweep time 120s and number of scans 4. Signals were quantified by measuring the total amplitude after correction of baseline and subtracting background signals. The mean value of three different samples of each subjects were used for statistical analysis.

HAECs were obtained from Clonetics (Clonetics Cell Systems, Germany), cultured in endothelial cell basal medium-2 supplemented with endothelial growth medium–SingleQuots as indicated by the manufacturer.

Effect of HDL on Endothelium-Dependent, NO-Mediated Vasodilation. The effect of HDL on endothelial NO production of intact vessels, i.e. the effect on endothelium-dependent, NO-mediated vasodilation, was examined by administration of increasing concentrations of HDL to aortic ring segments of C57/Bl6 mice (and as a control of eNOS^{-/-} mice). Vasorelaxation was determined as described in detail previously.²⁵ In brief, 3-mm ring segments of thoracic aortae were mounted in an organ bath (37°C) and gradually stretched

over one hour to a resting tension of 1.0 g. Maximal vasoconstriction was elicited by depolarization with 80 mM KCl and rings were washed thereafter. Following equilibration and after achieving a submaximal precontracted tone with phenylephrine (80 % of 80 mM KCl-elicited contraction) the responses of increasing concentrations of HDL were examined.

Effect of HDL on Endothelial Superoxide Production and NADPH Oxidase Activity.

The effect of HDL on endothelial superoxide production was assessed in TNF α -stimulated (100 U/ml, 24h) HAECs by using electron spin resonance (ESR) spectroscopy and the spin trap CM-H as described in detail previously.^{24, 26} The effect of HDL on the activity of NAD(P)H oxidase, a major endothelial cell oxidant enzyme system, was examined by ESR spectroscopy as described previously.^{24, 26}

Effect of HDL on Endothelial Repair Capacity of EPCs. EPCs were isolated and cultured as described in detail previously.^{24, 27-29} In brief, peripheral blood mononuclear cells were isolated by density gradient centrifugation with Biocoll (Biochrome, Berlin, Germany), and 10^7 cells were cultured on fibronectin-coated 6-well plates in endothelial cell basal medium-2 (containing 5 mmol/L glucose) supplemented with endothelial growth medium—SingleQuots exactly as indicated by the manufacturer except for hydrocortisone (Clonetics, Inc). After 4-day culture, non-adherent cells were removed by washing plates with PBS. Remaining cells were trypsinised and used for *in vivo* functional analysis.

EPCs from diabetic subjects were exposed to placebo (PBS), HDL from healthy subjects and HDL from diabetic subjects and *in vivo* re-endothelialisation capacity was assessed as described below. The effects of different HDLs were compared by using EPCs from the same diabetic subject. Male NRM1^{nu/nu} athymic nude mice, aged 7 to 10 weeks, were used to allow injection of human EPCs. Animals were anesthetized with ketamine (100 mg/kg IP)

and xylazine (5 mg/kg IP). Carotid artery electric injury was performed as described previously.^{24, 30, 31} In brief, the left common carotid artery was injured with a bipolar microregulator (ICC50, ERBE-Elektromedizin GmbH, Tuebingen, Germany). An electric current of 2 W was applied for 2 seconds to each millimetre of carotid artery over a total length of exactly 4 mm with the use of a size marker parallel to the carotid artery. EPCs (5×10^5 cells) were resuspended in 100 μ L of prewarmed PBS (37°C) and transplanted 3 hours after carotid injury via tail vein injection with a 27-gauge needle. The same volume of PBS was injected into control mice. Three days after carotid injury, endothelial regeneration was evaluated by staining denuded areas with 50 μ L of solution containing 5% Evans blue dye via tail vein injection as described previously.³² The re-endothelialised area was calculated as difference between the blue-stained area and the injured area by computer-assisted morphometric analysis. Of note, this model has been shown to allow accurate quantification of re-endothelialisation.^{24, 30}

NO production of EPCs was measured by ESR spectroscopy analysis using the spin-trap colloid Fe(DETC)₂ as described in detail previously.²⁴

Animals. The local animal research committee approved all animal protocols. C57/B16, eNOS^{-/-} and NRM1^{nu/nu} athymic nude mice were used as described above.

Lipid Peroxidation of HDL. HDL lipid peroxidation was measured by detecting the malondialdehyde (MDA) content in freshly isolated HDL, resulting from the decomposition of unstable peroxides, that was quantified colorimetrically following its controlled reaction with thiobarbituric acid using a TBARS Assay Kit (Cayman Chemical).³³⁻³⁵ Furthermore, HDL lipid oxidation was assessed by measurement of the anionic electrophoretic mobility of HDL as determined by electrophoresis on agarose gels and staining with sudan black

(Beckman) as described previously.^{34, 36, 37} Migration distance was measured by reference to origin.^{34, 36, 37} It has been described previously that oxidized lipoproteins, in particular oxidised HDL, increase their negative charge, and migrate faster in the agarose gel electrophoresis,^{34, 38, 39} that has been attributed, at least in part, to masking of positively charged lysine residues by lipid decomposition products^{38, 39} HDL lipid oxidation was determined in a randomly selected subgroup of study participants.

Myeloperoxidase(MPO)-catalyzed oxidation of HDL: HDL oxidation by myeloperoxidase-generated nitrating and chlorinating oxidants was carried out as described in detail previously.⁴⁰ In brief, HDL was resuspended in phosphate buffer (pH 7.4) containing 20mM sodium phosphate and 100 μ M diethylenetriaminepentaacetic acid (DTPA) at 37°C and for modification of HDL by the MPO-H₂O₂-chloride system the reaction mixture was supplemented with 50nM MPO, 0.1M NaCl and 125 μ M of H₂O₂. For modification of HDL by the MPO-H₂O₂-nitrite system the reaction mixture was supplemented with 50nM MPO, 100 μ M nitrite and 125 μ M of H₂O₂. Oxidative modification of HDL was initiated by adding MPO and terminated after 60 min by adding 5mM methionine.

Myeloperoxidase activity and content. HDL-associated myeloperoxidase (MPO) activity was measured as described in detail previously by UV spectrophotometry using guaiacol as the substrate in a randomly selected subgroup of study participants.⁴¹ Briefly, isolated HDL (100 μ g protein/well) was dissolved in 20mM phosphate buffer (pH 7.0) containing 0.34mM H₂O₂ and 200 μ M diethylene triamine penta-acetic acid. The reaction was initiated by addition of 14.4 mM guaiacol and the increase in absorbance at 470nm due to generation of guaiacol oxidation product was recorded at 25°C. Myeloperoxidase activity was calculated from the millimolar absorbance coefficient of 26.6mM⁻¹ · cm⁻¹ (at 470nm) for the diguaiacol oxidation

product and one unit of MPO activity was defined as the amount that consumes 1 μ mol of H₂O₂ per minute at 25°C. Myeloperoxidase activity was normalized per microgram of HDL protein.

Myeloperoxidase content of HDL (50 μ g protein) was determined by Western Blot analysis using a specific antibody to human MPO (Dako, Baar, Switzerland).

Binding of HDL to Endothelial Cells. Binding of HDL to human aortic endothelial cells was examined as described in detail previously.⁴² In brief, HDL was iodinated with Na¹²⁵I by the McFarlane monochloride procedure as modified for lipoproteins. Specific activities of approximately 300-700 cpm/ng of protein were obtained. Interactions of ¹²⁵I-HDL (5 μ g/ml, 50 min) with endothelial cells were examined and specific binding was calculated by subtracting the values of the non-specific binding from those of the total binding as described previously.⁴² Since large amounts of HDL were required for the endothelial binding studies, additional diabetic patients with the inclusion criteria of the present study and age- and sex-matched healthy subjects (n=7-8) were recruited for these measurements.

Measurement of Flow-Mediated, Endothelium-Dependent Vasodilatation (FDD).

Endothelium-dependent vasodilation of the radial artery and radial artery blood flow were examined as described in detail previously.^{43, 44} In brief, radial artery diameters were measured using high-resolution ultrasound (ASULAB). Then, an 8-minute wrist arterial occlusion was performed and FDD in response to reactive hyperemic blood flow was examined. All measurements were recorded and 2 investigators unaware of the interventions subsequently analyzed vessel diameters. This method is well established in our laboratory and has an excellent reproducibility and variability.^{43, 44}

Statistical Analysis. All data are expressed as mean \pm SD (1) For the statistical analysis a comparison of endothelial effects of HDL from healthy subjects and diabetic patients was performed by using the Mann-Whitney-U Test. A value of $P < 0.05$ (two-sided) was considered statistically significant. (2) Furthermore, a comparison of the changes of endothelial effects of HDL in diabetic patients (with metabolic syndrome) after ER-niacin versus placebo therapy was performed by using the Mann-Whitney-U Test. The primary end point for this study was the effect of HDL on endothelial cell nitric oxide production after therapy, which was used to determine the study size. The relevant alternative was a change of 20% of endothelial nitric oxide production. With the assumption of a common SD of 15%, a sample size of 30 patients randomized 1:1 was needed to have a power of >90% to reject the null hypothesis in favour of the alternative hypothesis with a 0.05 type I error.

The primary statistical analysis for the comparison of the changes of endothelial effects of HDL in diabetic patients (with metabolic syndrome) after ER-niacin versus placebo therapy was performed for the 30 patients that had completed the study. There were 3 patients (one in the placebo group and two patients in ER-niacin group) that discontinued therapy. A sensitivity analysis was performed assuming the “worst case scenario” as described in detail in the results section.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Participants. Baseline demographic and clinical characteristics of patients with type-2 diabetes and healthy subjects are shown in Table 1. Participants were included into the study between July 2006 and April 2009.

HDL from Diabetic Patients Loses Beneficial Effects on Endothelial NO Production and Endothelium-Dependent Vasodilation. HDL isolated from healthy subjects, but not HDL from type-2 diabetic patients, markedly stimulated endothelial cell NO production as detected by electron spin resonance spectroscopy analysis (Figure 1 A-B). Moreover, HDL from healthy subjects dose-dependently stimulated endothelium-dependent, NO-mediated vasodilation of intact vessels, an effect that was substantially impaired, when HDL from diabetic patients was examined (Figure 1 C,D). HDL had no effect on endothelium-dependent vasodilation in endothelial NO-synthase-deficient mice, indicating that HDL-induced vasorelaxation was eNOS-dependent (Figure 1 C,D).

HDL from Healthy Subjects, but not from Diabetic Patients Inhibits Endothelial Oxidant Stress. HDL isolated from HS substantially reduced TNF α -stimulated endothelial superoxide production and NAD(P)H oxidase activity as assessed by electron spin resonance (ESR) spectroscopy analysis (Figure 2 A-C), indicating a potent antioxidant effect of HDL from healthy subjects on the endothelium. Notably, however, HDL from diabetic patients had no significant inhibitory effect on endothelial cell superoxide production or NAD(P)H oxidase activity, indicating a loss of these antioxidant effects of HDL on the endothelium in diabetic patients (Figure 2 A-C).

HDL from Diabetic Patients has a Reduced Binding Capacity to Endothelial Cells as Compared to HDL from Healthy Subjects. The binding capacity of HDL to endothelial cells was analyzed by labelling HDL from healthy subjects and diabetic patients with Na¹²⁵I as described previously.⁴² Both, total and specific binding of HDL from diabetic patients to endothelial cells was significantly reduced as compared to HDL from healthy subjects, as shown in Figure 2 D.

HDL from Healthy Subjects, but not from Diabetic Patients Stimulates EPC-Mediated Endothelial Repair. *In vivo* endothelial repair capacity of EPCs from patients with type 2 diabetes was severely reduced as compared to healthy subjects (Figure 3 A,B), as demonstrated by transplantation of equal numbers of EPCs from diabetic and healthy subjects into nude mice with denuded carotid arteries and measurement of the re-endothelialised area by morphometric analysis three days after transplantation (Figure 3 A,B).

Notably, application of HDL (50 µg/ml, 60 min, 37°C) from healthy subjects, but not from diabetic subjects restored *in vivo* endothelial repair capacity of EPCs of diabetics (Figure 3 A,B), indicating a loss of the endothelial repair promoting effect of HDL in diabetic patients. For these studies HDL from diabetic patients and healthy subjects was added to EPCs derived from the same diabetic patient (Figure 3 A,B). HDL from healthy subjects also stimulated endothelial repair capacity of EPCs from healthy subjects (Supplementary Figure 1 A). Recent studies have suggested that a reduced NO production of EPC is critical for their impaired endothelial repair capacity in diabetic patients.^{24, 45} Notably, HDL from healthy subjects, but not HDL from diabetic patients stimulated NO production of EPCs (Figure 3 C) suggesting that HDL may promote EPC-mediated endothelial repair by stimulating NO production. Moreover, inhibition of NO production in EPCs from diabetic patients (by 1 mM L-NAME) prevented the effects of HDL from healthy subjects on EPC-mediated endothelial

repair (Supplementary Figure 1 B), further supporting the concept that the effect of HDL on EPC-mediated endothelial repair is, at least in part, mediated by stimulating NO production of EPCs.

Increased Lipid-Peroxidation of HDL from Diabetic Patients. Measurement of both, the MDA concentration and electrophoresis mobility of HDL suggested a substantially increased lipid peroxidation of HDL isolated from diabetic patients as compared to HDL from healthy subjects (Figure 4 A,B). Myeloperoxidase-dependent oxidation of HDL has been suggested as a potential mechanism to alter the effects of HDL on cholesterol-efflux from macrophages.¹⁶ Moreover, myeloperoxidase levels have been found to be closely associated with endothelial dysfunction.⁴⁶ We therefore determined the effect of myeloperoxidase-dependent oxidation of HDL on the effects of HDL on endothelial NO production.

Effect of Myeloperoxidase-dependent Oxidation of HDL on the Capacity of HDL to stimulate Endothelial NO Production. Exposure of HDL from healthy subjects to both MPO-derived oxidants, chlorinating and nitrating oxidant species, rapidly impaired the capacity of HDL to stimulate endothelial NO production (Fig 4 E). Moreover, HDL-associated myeloperoxidase activity and protein content were significantly increased in diabetic patients as compared to healthy subjects (Figure 4 C,D), compatible with the concept that MPO-derived oxidants may contribute to altered endothelial effects of HDL in diabetic patients.

Endothelium-dependent Vasodilation in Healthy Subjects and Diabetic Patients. Flow-mediated, endothelium-dependent vasodilation of the radial artery was significantly impaired

in diabetic patients (n=33) as compared to healthy subjects (n=10; FDD: 5.1 ± 1.9 vs. 12.0 ± 2.3 %; $P < 0.0001$).

ER-Niacin Therapy Improves Endothelial-protective Effects of HDL in Diabetic Patients. ER-niacin therapy increased HDL levels in patients with diabetes (Table 2). Importantly, the capacity of HDL to stimulate endothelial cell NO-production was increased in diabetic patients after ER-niacin therapy as compared to placebo treatment (Figure 5 A). The primary statistical analysis was performed for the 30 patients that had completed the study. There were 3 patients (one in the placebo group and two patients in ER-niacin group) that discontinued therapy. A sensitivity analysis was performed for the primary outcome assuming the “worst case scenario”, i.e. the “best” treatment effect was assumed for the missing follow-up value of the patient in the placebo group and the “worst” treatment effect was assumed for the two missing follow-up values of the patients in the active treatment group. This sensitivity analysis indicated that there still remained a significant effect of active treatment on HDL function, i.e. HDL-stimulated endothelial NO production ($P=0.019$). Furthermore, HDL isolated from diabetic patients after ER-niacin treatment exerted a more potent inhibitory effect on endothelial superoxide production and NAD(P)H oxidase activity, suggesting that ER-niacin therapy increased the endothelial antioxidant effects of HDL (Figure 5 B-D). Furthermore, HDL isolated from ER-niacin-treated patients stimulated endothelium-dependent vasodilation of intact mouse aortic rings significantly better as compared to HDL isolated from placebo-treated diabetic patients (Supplementary Figure 2). These findings suggest that ER-niacin therapy does not only increase HDL-levels, but potentially more important, exerts a beneficial effect on endothelial-protective properties of HDL in diabetic patients.

ER-Niacin Therapy Restores the Effect of HDL on *in vivo* Endothelial Repair Capacity of EPCs in Diabetic Patients. HDL isolated from diabetic patients after a 3-months treatment with ER-niacin had an increased effect on EPC *in vivo* endothelial repair-capacity (Figure 6 A,B). Notably, in diabetic patients after niacin therapy HDL had an improved effect on endothelial progenitor cell NO production as determined by ESR spectroscopy (Figure 6 C).

ER-Niacin Therapy and Lipid Peroxidation of HDL in Diabetic Patients. The measurement of electrophoresis mobility of HDL suggested a substantial reduction of lipid peroxidation of HDL in diabetic patients after extended-release niacin therapy as compared to placebo treatment (Figure 7 A). The measurements of MDA content as an indicator of lipid oxidation revealed a lower MDA content after ER-niacin therapy, however, the comparison of the changes of the MDA content between the ER-niacin and placebo group did not reach statistical significance (Figure 7 B). We further determined HDL-associated activity and content of myeloperoxidase, that may promote lipid oxidation, before and after ER-niacin or placebo therapy.

HDL-bound Myeloperoxidase Activity and Content are Reduced after ER-Niacin Therapy: HDL-associated myeloperoxidase activity and content were reduced after ER-niacin therapy as compared to placebo therapy, compatible with the concept that ER-niacin therapy may reduce the detrimental impact of myeloperoxidase-derived oxidants on HDL in diabetic patients (Figure 7 C,D).

ER-Niacin Therapy Improves Endothelium-Dependent Vasodilatation in Diabetic

Patients. After 3 months of treatment with extended-release niacin, but not after placebo therapy, endothelium-dependent vasodilation was substantially improved in diabetic patients (Figure 8). This was not due to differences in radial artery blood flow during reactive hyperemia. Radial artery blood flow at maximal reactive hyperemia was similar in both groups before (placebo vs. ERN group, 98 ± 11 versus 87 ± 9 mL/min; $P=n.s.$) and after placebo or niacin therapy (placebo vs. ERN group, 95 ± 11 versus 99 ± 15 mL/min; $P=n.s.$).

Discussion

The present study demonstrates that HDL from patients with type-2 diabetes loses the capacity to directly stimulate endothelial nitric oxide production and to reduce endothelial oxidant stress, that is in marked contrast to the effects observed with HDL from healthy subjects. Furthermore, HDL from healthy subjects, but not from diabetic patients promoted *in vivo* endothelial repair capacity of early endothelial progenitor cells. More importantly, ER-niacin therapy not only increased HDL plasma levels in diabetic patients, but also improved endothelial-protective properties of HDL, i.e. HDL isolated from diabetic patients after ER-niacin therapy had an improved capacity to stimulate endothelial NO production and EPC-mediated endothelial repair and to exert antioxidant effects on endothelial cells. This was associated with an improvement of endothelium-dependent vasodilation in diabetic patients after ER-niacin therapy. ER-niacin therapy may therefore represent a promising strategy not only to increase HDL plasma levels, but likely more important, to restore direct endothelial-protective functions of HDL.

Reduced plasma levels of HDL are associated with an increased risk of coronary disease and cardiovascular events, even in patients with low LDL levels on statin therapy.³ This has been attributed, at least in part, to HDL-mediated vasoprotective effects. Notably, reconstituted HDL or HDL isolated from healthy subjects has been shown to stimulate endothelial cell NO production, to improve endothelium-dependent vasodilation⁴⁻⁷ and to exert beneficial effects on endothelial progenitor cell-mediated vascular repair,^{9, 10} however, these effects were observed by using either reconstituted HDL or HDL from healthy subjects.

Notably, more recently it has been suggested that the effects of HDL can be highly heterogeneous. Modification of HDL by pathophysiological concentrations of myeloperoxidase has been observed to impair the effect of HDL on cholesterol efflux from

macrophages by the ATP-binding cassette transporter A-1 (ABCA-1) pathway.¹⁶ Moreover, HDL from patients with coronary disease exerted a pro-inflammatory rather than an anti-inflammatory effect, that was partially attenuated in patients on statin therapy.¹⁷ In addition, the capacity of HDL to counteract the oxidized LDL-induced impairment of vascular relaxation was reduced in type-2 diabetic patients.¹⁸

In the present study we demonstrate that HDL from type-2 diabetic patients loses its capacity to directly stimulate endothelial NO production, in contrast to HDL isolated from healthy subjects. This is demonstrated both, by a lack of HDL from diabetic patients to stimulate endothelial cell NO production as well as by a loss of the effect of HDL on NO-mediated vasodilation of intact arterial segments. Moreover, we and others have observed that eNOS-derived NO production plays a major role for *in vivo* re-endothelialisation capacity of endothelial progenitor cells,²⁴ and HDL has been suggested to promote EPC-mediated endothelial repair.¹⁰ In the present study we demonstrate that the beneficial effects of HDL on EPC-mediated endothelial repair are markedly impaired in diabetic patients, likely at least in part related to a reduced effect of HDL from diabetics on NO production of EPCs.

The present findings therefore support the concept that pharmacologic HDL-raising interventions should be examined with regard to their ability to restore vasoprotective properties of HDL. In fact, recent studies evaluating HDL-raising interventions have yielded mixed results.^{11,12}

The mechanisms underlying the loss of beneficial effects of HDL on endothelial NO production in diabetic patients are likely multifactorial, and may include oxidative modification of HDL, changes in HDL composition, and potentially altered endothelial binding of HDL. In this respect, we have observed an increased lipid peroxidation of HDL from diabetic patients in the present study. Of note, myeloperoxidase has been suggested to modify HDL and its capacity to promote cholesterol efflux from macrophages.¹⁶ Moreover,

myeloperoxidase serum levels have been shown to be independently associated with endothelial dysfunction,⁴⁶ and to predict risk of coronary disease.⁴¹ Notably, in the present study we have observed that exposure of HDL from healthy subjects to both, myeloperoxidase-generated nitrating and chlorinating oxidants rapidly impaired its capacity to stimulate endothelial NO production, compatible with the notion that myeloperoxidase-derived oxidants may exert effects on HDL to prevent its endothelial NO stimulating properties. Of note, an increased binding affinity of HDL for myeloperoxidase after myeloperoxidase-mediated oxidation of HDL has recently been reported, that may lead to a vicious cycle of MPO transport and MPO-dependent oxidation of HDL.⁴⁷ We have therefore determined HDL-associated MPO activity and content. Notably, both HDL-associated MPO activity and content were increased in HDL from diabetic patients as compared to healthy subjects, further compatible with a role of myeloperoxidase for altered endothelial effects of HDL from diabetic patients.

Furthermore, our endothelial binding studies suggest that HDL from diabetic patients has a reduced binding capacity towards endothelial cells, which may contribute to altered endothelial effects of HDL from diabetics. However, this will likely not completely explain the lack of effect of HDL from diabetic patients on endothelial NO production, because there was still detectable specific endothelial binding of HDL from diabetic patients, and a significant NO-stimulating effect of HDL from healthy subjects was observed at low HDL concentrations.

Of note, in the present study diabetic patients were on statin therapy, suggesting, that this is not sufficient to restore an endothelial-protective phenotype of HDL. This is in line with a recent study by Ansell et al., suggesting that statin therapy attenuates the pro-inflammatory effect of HDL from patients with coronary disease, however, is not sufficient to lead to HDL with anti-inflammatory properties as is observed in healthy subjects.¹⁷ Therefore, it is of

particular interest how vasoprotective properties of HDL can be further improved in patients at risk.

Importantly, the present study demonstrates for the first time that ER-niacin therapy promotes endothelial-protective properties of HDL in type-2 diabetic patients. HDL from diabetics after ER-niacin therapy, but not after placebo treatment, had an improved capacity to stimulate endothelial NO production, to promote EPC-mediated endothelial repair and to inhibit endothelial cell oxidant stress. The mechanisms by which niacin therapy exerts these effects on the vascular properties of HDL remain to be further evaluated, however, may include a reduced lipid oxidation of HDL after ER-niacin therapy. In particular, HDL-associated myeloperoxidase activity and content were reduced after ER-niacin therapy that may contribute to a reduced oxidation of HDL. The mechanisms whereby niacin may exert these antioxidant effects remain to be further determined. Interestingly, recent studies have suggested an upregulation of peroxisome proliferator-activated receptor- γ (PPAR γ) in monocytes/macrophages and adipocytes after niacin therapy,^{48, 49} and PPAR γ agonism has been observed to inhibit hypercholesterolemia-induced leukocyte myeloperoxidase activation,⁵⁰ raising the possibility that this may represent a potential pathway contributing to antioxidant effects of niacin therapy. Moreover, Green et al. have recently suggested that niacin therapy may reverse changes of the HDL proteome as observed in six patients with coronary disease, representing another potential explanation for different vascular effects of HDL after niacin therapy.⁵¹ In particular, a partial reversal of changes of the HDL-associated proteins apoE, apoCII, apoJ, apoF, and PLTP in coronary disease has been observed after niacin therapy, however, the underlying mechanisms and functional implications remain to be further determined.⁵¹

Notably, ER-niacin has been shown to reduce the progression of carotid intima media thickening over time in patients with coronary disease on statin therapy and low HDL

levels.⁵² Moreover, the combination of niacin and statin therapy has been suggested to exert beneficial clinical and coronary angiographically measurable effects in a moderate-sized study in patients with coronary disease and low HDL levels.⁵³

The present study provides a novel explanation for potential beneficial effects of ER-niacin therapy on the progression of atherosclerosis, i.e. the restoration of endothelial-protective properties of HDL. Currently, two large-scale studies examine the effect of ER-niacin therapy on cardiovascular events in high-risk patients (HPS-THRIVE, AIM-HIGH). Whether CETP inhibition exerts a similar effect on vascular properties of HDL remains to be determined.

Study Limitations: Given that most study participants of the present study were male, we cannot exclude that there may be different endothelial responses of HDL from female patients as compared to male patients with and without ER-niacin therapy. A subgroup analysis did not reveal significant differences between the endothelial responses of HDL from male and female patients, however, this will have to be analysed further in future studies.

The present study has included healthy subjects and patients with type-2 diabetes with reduced HDL levels (meeting the criteria for metabolic syndrome). As shown in Table 1, the characteristics of diabetic patients and healthy subjects were significantly different in terms of BMI, waist circumference, LDL and HDL cholesterol levels. Therefore, the differences in study outcomes may not necessarily be related to the fact that one group had diabetes and the other did not, but may also be related to the above factors, i.e. to differences in adiposity and lipid levels. The rationale for examining patients with both, diabetes and the components of the metabolic syndrome (i.e. increased weight circumference, elevated triglycerides, low HDL) was that these patients are frequently considered for HDL-raising therapies, given their low HDL levels and substantially increased cardiovascular risk. Moreover, the patients were on medication, i.e. statin therapy, which may have an impact on endothelial effects of HDL.

Indeed, a recent study has suggested that statin therapy reduces pro-inflammatory effects of HDL in patients with coronary disease.¹⁷ However, the rationale for including patients on statin therapy in the present study was that HDL-targeted treatment approaches are currently explored on top of statin therapy, which represents most frequently the first-line treatment.

In summary, the present study provides novel evidence suggesting that HDL from diabetic patients, in contrast to HDL from healthy subjects, has markedly impaired endothelial-protective effects. Importantly, ER-niacin therapy substantially improved vasoprotective properties of HDL and endothelial function in diabetic patients on statin therapy. As recent studies, evaluating HDL-raising interventions, have yielded mixed results,^{11, 12} circulating HDL cholesterol levels alone likely do not represent an adequate measure of therapeutic efficacy and indexes of HDL functionality are urgently needed for assessment of the potential of HDL-targeted therapies to exert vasoprotective effects.

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Disclosures

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Figure legends

Figure 1. **A:** Effect of HDL (50 $\mu\text{g/ml}$, 60 min, 37°C) from healthy subjects ($n=10$) and diabetic patients ($n=33$) on endothelial cell nitric oxide production as determined by ESR spectroscopy analysis (representative spectra are shown in figure 1 **B**.) **(C)** Endothelium-dependent relaxation of aortic rings of wild type mice in response to increasing concentrations of HDL isolated from healthy subjects ($n=5$) or diabetic patients ($n=5$) are shown. The P-value indicates the statistical comparison via the Mann-Whitney test between the effect of 100 μg HDL from healthy subjects with the effect of 100 μg HDL from the patient group on endothelium-dependent vasodilation. **(D)** Maximum endothelium-dependent relaxations in wild type and eNOS^{-/-} -mice in response to HDL from healthy subjects and diabetic patients are shown ($n=5$).

Figure 2. ESR spectroscopy analyses of the effect of HDL (50 $\mu\text{g/ml}$, 60 min, 37°C) isolated from healthy subjects ($n=10$) and diabetic patients ($n=33$) on endothelial oxidant stress, i.e. TNF- α stimulated endothelial cell superoxide production (**A**) and NAD(P)H oxidase activity (**B**) are shown. **C:** Representative ESR spectra of endothelial superoxide production in response to HDL from a healthy subjects and a diabetic patient are shown **(D)** Total and specific endothelial binding of radioactively labelled ¹²⁵I-HDL from healthy subjects and diabetic patients to human aortic endothelial cells. Endothelial cells were incubated with ¹²⁵I-HDL (5 $\mu\text{g/ml}$) in the absence (total) or in the presence of an excess of unlabelled autogenic HDL (non-specific). Specific binding of HDL was calculated by subtracting the values of non-specific binding from the total binding ($n=7-8$).

Figure 3. Effect of HDL isolated from healthy subjects and diabetic patients on endothelial repair. **A,** Re-endothelialised area at day 3 after carotid injury in nude mice with

transplantation of early EPCs from healthy subjects ($n=10$), diabetic patients ($n=33$) and from diabetic patients co-incubated with HDL ($50 \mu\text{g/ml}$, 60 min , 37°C) from healthy subjects or diabetic patients (each 5×10^5 EPCs). **B**, Representative photographs of re-endothelialised area. **C**. Effect of HDL isolated from healthy subjects and diabetic patients on nitric oxide (NO) production of EPC as determined by ESR spectroscopy.

Figure 4. **A** and **B** show levels of lipid oxidation of HDL from healthy subjects or diabetic patients as determined by an electrophoresis mobility assay and MDA measurements ($n=5-10$ experiments for each column). **C** and **D** show content and activity of myeloperoxidase of HDL from healthy subjects or diabetic patients ($n=5-10$). **E** shows the effect of myeloperoxidase-derived chlorinating or nitrating oxidants on the capacity of HDL from healthy subjects to stimulate endothelial NO production ($n=4-5$).

Figure 5. Effect of ER-niacin therapy or placebo on endothelial-protective properties of HDL in diabetic patients. ESR spectroscopy analysis of endothelial NO bioavailability (**A**) and superoxide production (**B**) and NAD(P)H oxidase activity (**C**) of endothelial cells (TNF- α stimulated) in response to HDL ($50 \mu\text{g/ml}$, 1 h , 37°C) from diabetic patients before and after 3 months ER-niacin therapy ($n=15$) or placebo ($n=15$). Representative ESR spectra of superoxide production are shown (**D**). The P-values relate to the statistical analyses of changes (i.e. treatment effects) of the ER-niacin vs. the placebo group.

Figure 6. **A**, Re-endothelialised area at day 3 after carotid injury in nude mice with transplantation of EPCs from diabetic patients, co-incubated with HDL ($50 \mu\text{g/ml}$, 1 h , 37°C) from diabetics before and after 3 months ER-niacin therapy ($n=15$) or placebo ($n=15$). EPCs of the same subjects were used for before-and-after-treatment analyses. **B**, Representative

photographs of the effect of HDL on vascular repair. **C.** Effect of HDL isolated from diabetic patients before and after niacin or placebo therapy on endothelial progenitor cell NO production as determined by ESR spectroscopy. The P-values relate to the statistical analyses of changes (i.e. treatment effects) of the ER-niacin vs. the placebo group.

Figure 7. **A** and **B** show levels of lipid oxidation of HDL from diabetic patients before and after 3 months of ER-niacin or placebo therapy ($n=3-7$ experiments for each column). **C** and **D** show the content and activity of myeloperoxidase of HDL from diabetic patients before and after niacin or placebo therapy ($n=5$). The P-values relate to the statistical analyses of changes (i.e. treatment effects) of the ER-niacin vs. the placebo group.

Figure 8. FDD (change in radial artery diameter, %) after wrist occlusion during reactive hyperemia in diabetic patients before and after 3 months of ER-niacin therapy ($n=15$) or placebo ($n=15$). The P-values relate to the statistical analyses of changes (i.e. treatment effects) of the ER-niacin vs. the placebo group.

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