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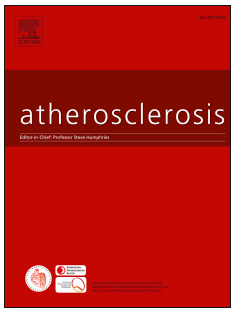

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Remarkable Quantitative and Qualitative Differences in HDL after Niacin or Fenofibrate Therapy in Type 2 Diabetic Patients

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ABSTRACT

HDL-increasing drugs such as fenofibrate and niacin have failed to decrease the cardiovascular risk in patients with type 2 diabetes. Drug-mediated quantitative and qualitative HDL modifications could be involved in these negative results. To evaluate the quantitative and qualitative effects of niacin and fenofibrate on HDL in patients with type 2 diabetes, a prospective, randomised controlled intervention trial was conducted. Thirty type 2 diabetic patients with low HDL were randomised to receive either fenofibrate (FFB) or niacin + laropiprant (ERN/LPR) as an add-on to simvastatin treatment for 12 weeks according to a crossover design. At the basal point and after each intervention period, physical examinations and comprehensive standard biochemical determinations and HDL metabolomics were performed. Thirty nondiabetic patients with normal HDL were used as a basal control group. ERN/LRP, but not FFB, significantly increased HDL cholesterol. Neither ERN/LRP nor FFB reversed the HDL particle size or particle number to normal. ERN/LRP increased apoA-I but not apoA-II, whereas FFB produced the opposite effect. FFB significantly increased Preβ1-HDL, whereas ERN/LRP tended to lower Preβ1-HDL. CETP and LCAT activities were significantly decreased only by ERN/LRP. PAF-AH activity in HDL and plasma decreased with the use of both agents. Despite their different actions on antioxidant parameters, none of the treatments induced detectable antioxidant improvements.

ERN/LRP and FFB had strikingly different effects on HDL quantity and quality, as well as on HDL cholesterol concentrations. When prescribing HDL cholesterol increasing drugs, this differential action should be considered.

Key words: HDL; niacin; fenofibrate; Preβ1-HDL; PON1; PON3; CETP; LCAT; PAF-AH; Type 2 diabetes; HDL particle size; nuclear magnetic resonance

1. Introduction

Cardiovascular (CV) diseases are responsible for approximately 50% of deaths in patients with type 2 diabetes [1]. In the presence of additional CV risk factors, achieving a low density lipoprotein (LDL) concentration below 70 mg/dl is recommended [2]. Even if the LDL target is achieved, an important residual risk remains. A portion of this residual risk has been attributed to lipid profile alterations, as well as plasma LDL concentrations [3]. Patients with type 2 diabetes usually have profound lipid metabolism derangement, which is characterised by low high density lipoprotein (HDL) and high triglyceride concentrations. This lipid pattern is referred to as atherogenic dyslipidemia because of its high vascular damaging capacity. The inverse association between circulating HDL cholesterol concentrations and CV disease risk is unquestionable [4]. In many epidemiological studies, HDL cholesterol below 40 mg/dl in men and 45 mg/dl in women has been associated with an increased CV disease risk [2]. Recent data from the "Emerging Risk Factors Collaboration" confirmed that ~~a difference in~~ HDL cholesterol is inversely associated to coronary heart disease after adjusting for lipid and non-lipid risk factors [5].

Despite this strong epidemiological association, increasing HDL cholesterol by medications has not produced a beneficial impact on CV disease risk. In recent years, clinical outcome intervention trials using fibrates, niacin and cholesteryl ester transfer protein (CETP) inhibitors have had negative results [6]. Among the fibrate intervention trials, only the VA-HIT study using gemfibrozil showed a 22% relative CV disease risk reduction associated with a 6% increase in HDL cholesterol [7]. More recent studies using fenofibrate (FIELD and ACCORD) [6, 8] did not show a beneficial effect, albeit post hoc analyses suggested a marginal benefit in the atherogenic dyslipidemia subgroup. Similarly, two studies that used niacin as an add-on to statin treatment, AIM-

HIGH and HPS2-THRIVE, were prematurely stopped due to a lack of efficacy [9, 10]. In all of these trials, the effect on HDL cholesterol concentrations was relatively poor, with mean increases ranging from 0% to 6%. The failure of the fibrate and niacin trials has been attributed to a lack of effect on lipid parameters or a poor study design, among other reasons. Beyond these circumstances, the complex composition and metabolism of HDL particles must be considered. Data from proteomic and lipidomic studies have shown the heterogeneity of this lipoprotein family, which is involved in many biological functions [11-~~14~~15]. ~~The fact that more than 300 proteins are associated with HDL underlines the complexity of the composition of HDL~~. Moreover, HDL has considerable plasticity and is capable of changing its composition according to the environmental needs. ~~In a proinflammatory situation, HDL is loaded with inflammatory mediators that can lead to non-protective or even proatherogenic particles [15].~~ although reverse cholesterol transport is considered to be the key HDL antiatherogenic function, other biological effects of HDL are equally important, including its anti-inflammatory, endothelial protective, and antioxidant capacities [16]. The cholesterol content in HDL is only a subrogated marker of the HDL particle concentration and has a weak correlation with HDL functions [17]. The anti-inflammatory, antioxidant, and endothelial protective or antiapoptotic effects of HDL seem to be more related to the HDL particle shape, size, number and composition. All of these characteristics are altered by pathological conditions such as type 2 diabetes mellitus (T2DM) [18]. Many efforts have been made to evaluate the clinical impact of HDL function rather than HDL cholesterol concentrations. Recently, the HDL cholesterol efflux capacity was observed to be a better indicator of HDL CV protection than HDL cholesterol [19]. Despite this evidence, HDL cholesterol concentrations remain the primary treatment determinant, and the efficacy of medication is assessed by its capacity to increase HDL cholesterol.

~~In this study, we evaluated the effects of ERN/LRP and FFB on HDL particle size distribution and composition, as well as the metabolic determinants of HDL in patients with T2DM.~~

In this study, we hypothesized that global HDL particle alterations of T2DM patients are reversed by neither ERN/LRP nor FFB despite their HDL cholesterol increasing effect and that both drugs impact differently in HDL particle size distribution, composition and HDL metabolic determinants in these patients.

2. Patients and methods

2.1 Subjects and design of the study

Thirty type 2 diabetic patients, 19 male and 11 female, ranging in age from 30 to 70 years old and with HDL not exceeding 50 mg/dl in men or 60 mg/dl in women were recruited. This HDL cut off points were selected to avoid the impact of genetic factors associated to high HDL values. The exclusion criteria were as follows: smoker, diagnosed with diabetes less than three months before, triglyceride levels above 400 mg/dl, glycated haemoglobin higher than 9%, albuminuria above 300 mg/mg creatinine, chronic kidney disease (estimated glomerular filtration rate <30 ml/min/1.73 m^2), advanced retinopathy, neuropathy, cardiovascular disease in the last three months, chronic liver insufficiency, neoplastic disease or any chronic or incapacitating disease. The control group consisted of 30 age- and gender-matched subjects without diabetes and with HDL cholesterol higher than 40 mg/dl for men or 50 mg/dl for women. After a 6-week lipid-lowering drug wash-out, the patients with type 2 diabetes were randomly distributed into two groups. One group received 20 mg simvastatin plus 145 mg fenofibrate, and the other group received 20 mg simvastatin plus 2 g niacin plus laropiprant for a 12-week period. After this intervention period, the patients followed a

new 6-week lipid-lowering drug wash-out; subsequently, they were shifted to the other lipid-lowering drug, in a crossover design, for a 12-week period (Figure 1). Physical examinations, anthropometry and blood extraction for standard biochemical and metabolic tests were obtained at the basal point and after each intervention period in the type 2 diabetes group and at the basal point in the control group. All of the study investigations were conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Ethic Committees of the recruiting hospitals. All of the subjects provided their written informed consent before participating in the study.

2.2. Sample collection and storage

At the time points indicated in the flow chart (Figure 1), fasting blood samples were collected in serum tubes with EDTA and were centrifuged immediately at 1500 *g* for 15 min at 4ºC. Aliquots of plasma and serum were stored at -80ºC until the analyses were performed (except for preβ1-HDL).

2.3. Standard lipid analyses

Biochemical parameters, lipids, apolipoproteins, fructosamine and homocysteine were measured using colourimetric, enzymatic and immunoturbidimetric assays (Spinreact, SA, Spain; Wako Chemicals GmbH, Germany; Polymedco, NY) adapted to a Cobas Mira Plus autoanalyser (Roche Diagnostics, Spain) [20-22].

Enzymes and protein concentrations are outlined in the supplemental materials.

2.4. Preβ1-HDL measurements

To determine preβ1-HDL measurements, plasma samples were immediately placed on ice in a 50% sucrose solution. Preβ1-HDL was analysed using a quantitative ELISA (Daichii, Japan).

2.5. HDL isolation using ultracentrifugation

Total HDL was isolated from plasma using sequential preparative ultracentrifugation (uc) at 1.21 mg/dl density according to previously described techniques [23]. Ultracentrifuged HDL (ucHDL) fractions were stored at -80ºC until biochemical studies were performed.

2.6. Plasma and HDL oxidation status

The OxyStat (Biomedica, Wien) colorimetric assay was used for the quantitative determination of lipid peroxides in apoB-depleted plasma after the precipitation of β-lipoproteins using phosphotungstic acid and magnesium ions (Roche Diagnostics). The results are expressed as μmol lipoperoxides/mg apoA-I.

Serum paraoxonase 1 (PON1) and paraoxonase 3 (PON3) concentrations were determined using an in-house ELISA and rabbit polyclonal antibodies generated against synthetic peptides with sequences specific for mature PONs. The employed peptides were CRNHQSSYQTRLNALREVQ (specific for PON1) and CRVNASQEVEPVEPEN (specific for PON3). The details of these methods have been previously reported [24]. Serum PON1 lactonase activity was analysed by measuring 5-thiobutyl butyrolactone (TBBL) hydrolysis, as previously described [25, 26].

HDL antioxidant activity was determined by conjugated diene formation by incubating the patient's HDL (0.1 mg/ml apoA1) with human LDL (0.1 mg/ml apoB, obtained from a pool of normolipidemic individuals) in the presence of 2.5 μmol/L CuSO4.

Continuous monitoring at an absorbance of 234 nm was performed in a microplate reader (BioTek Synergy, Winooski, VT, USA) at 37°C for 4 h. The kinetics of LDL in the LDL+HDL incubations were calculated by subtracting the kinetics of HDL incubated without LDL; the lag phase was calculated as previously described [27].

2.7. HDL composition analyses

In the ucHDL fraction, cholesterol, triglyceride, total protein, phospholipid, apolipoprotein AI (apoA-I) (Roche Diagnostics), apolipoprotein A-II (apoA-II), apolipoprotein E (apoE), apolipoprotein CII (apoC-II), and apolipoprotein CIII (apoC-III) (Kamiya Biomedical Company) contents were quantified using enzymatic and nephelometric assays adapted to a BM/HITACHI 911 autoanalyser (Spinreact S.A.U., Spain).

2.8. HDL analyses by 2D diffusion-ordered ^{1}H NMR spectroscopy (DOSY)

The ucHDL fraction samples were analysed using nuclear magnetic resonance (NMR) spectroscopy and a modified existing protocol [28]. The ^{1}H NMR spectra were recorded using a BrukerAvance III spectrometer at 310 K. We used the double stimulated echo (DSTE) pulse program with bipolar gradient pulses and a longitudinal eddy current delay (LED). The DSTE methyl signal was fitted with one lorentzian function to obtain the averaged diffusion coefficient of the lipoprotein particles. The hydrodynamic radii of the lipoprotein fractions were extracted from the Stokes-Einstein equation. Further details about the ucHDL NMR feature extraction and HDL particle size distribution and number calculations are outlined in the supplemental material.

2.9. Statistical analysis

Normal distributed data are shown as the mean ± SD values, and non-normal distributed data are shown as the median (interquartile range). We performed two different statistical tests to detect differences between the studied variables. A statistical Mann-Whitney U test was performed to identify significant differences between the control group and the group comprising patients with type 2 diabetes, followed by a Wilcoxon signed-rank test to evaluate the treatment effects for paired samples. We performed alpha corrections due to multiple testing by multiplying the p value by the number of related variables tested (lipids, enzymes, oxidation, HDL subclasses). We excluded any carryover effect by the Fleiss method. There were no significant differences (by t test) in the results obtained in any of the variables after the same treatment, regardless of the intervention order [29]. Subsequently, the data of the two sequences were combined and analysed as described in the design section. The analyses were performed using SPSS software (IBM SPSS Statistics, version 20). $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Considerable differences in HDL components and metabolic determinants between healthy and T2DM individuals and after both treatments

In **Table 1,** we show the lipid metabolism and oxidation parameters and HDL subclass particle numbers in both T2DM patients and controls. As expected, the patients with T2DM had lower HDL cholesterol and ApoA1. They had higher preβ1-HDL and CETP activity and lower PON1. The anthropometric and clinical characteristics of these groups are presented in **Supplemental Table 1S**.

Because of the side effects of the medications, one patient withdrew from the study during the fenofibrate treatment, and four patients withdrew during the niacin treatment. **Table 2** shows the basal and post FFB and ERN/LRP treatment values of lipid metabolism and oxidation parameters and HDL subclass particle numbers in T2DM patients. Only ERN/LRP significantly increased HDL cholesterol and apoA-I, whereas FFB increased apoA-II concentrations. The effect on preβ1-HDL was also divergent between the treatments; FFB increased preβ1-HDL, whereas ERN/LRP tended to decrease its concentration. FFB and ERN/ LRP also had significantly different effects on LOOH in HDL. The LCAT mass was reduced by both treatments, whereas only ERN/LRP treatment significantly decreased LCAT activity. PAF-AH activity was also decreased both in the plasma and HDL by both treatments. CETP activity was significantly reduced by ERN/LRP but was not modified by FFB. ERN/LRP increased the number of large HDL particles, whereas FFB decreased the number of medium size HDL particles. To better display these results, the main differences are shown in **Figure 2**.

Supplemental Table 2S shows anthropometric and clinical data at similar time points. **Supplemental Table 3S** shows the basal HDL composition in controls and T2DM

patients. HDL from T2DM patients had less cholesterol and apoE and more triglycerides and apoC-III

Table 4S shows the HDL composition at baseline and post ERN/LPR and FFB treatments in T2DM patients. ERN/LRP, but not FFB, increased the cholesterol content. FFB increased ApoA-II and reduced ApoC-III. None of the treatments affected the percentage of ApoE in HDL.

3.2. HDL particle size and number alterations were not fully reversed by treatment

The total number of HDL particles was lower in T2DM patients (P=0.009). The difference was primarily due to the medium-sized HDL particles (P=0.004) (**Table 1**). The total HDL particle number was not significantly modified by any of the treatments. Niacin did not produce any significant change in the HDL particle number, whereas FFB decreased the number of medium-sized HDL particles (P=0.044) (**Table 2**).

Figure 3A shows the NMR spectrum of large, medium and small HDL subclasses. **Figure 3B** shows the NMR spectrum of HDL subclasses for the control and T2DM subjects at the basal point and after both treatments. The HDL spectra of medium and large HDL particles were different between controls and T2DM and were not reversed to normal after any treatment. No differences in the NMR spectrum of small HDL were observed. **Figure 3C** shows the average HDL particle sizes, confirming that the HDL from T2DM patients is smaller than that of controls, and neither FFB nor ERN/LRP fully correct this alteration. The mean radius for the healthy group (4.7 nm) was higher than that of the T2DM group (4.5 nm) (P=0.002). FFB tended to shift the distribution towards smaller particles, whereas RN/LRP treatment increased the relative concentration of the medium HDL subclass, which consequently, although not significantly, approached the healthy state. The effects of different treatments on the mean HDL radius were significantly different (P=0.042).

3. Results

3.1. Baseline differences

The anthropometric and clinical characteristics of these groups are presented in **Supplemental Table 1S**. In **Table 1**, we show the lipid metabolism and oxidation parameters and HDL subclass distribution in both T2DM patients and controls. As expected, the patients with T2DM had lower HDL cholesterol and ApoA1. The total number of HDL particles was lower in T2DM patients (P=0.009). The difference was primarily due to the medium-sized HDL particles (P=0.004) **(Table 1)**. They had higher preβ□-HDL and CETP activity and lower PON1. HDL from T2DM patients had less cholesterol and apo E and more triglycerides and apoC-III **(Supplemental Table 2S)**. Because of the side effects of the medications, one patient withdrew from the study during the fenofibrate treatment, and four patients withdrew during the niacin treatment. The anthropometric and clinical characteristics of these groups are presented in **Supplemental Table 3S.**

3.2. Changes induced by fenofibrate

Despite a significant decrease in plasma TG, FFB did not increase HDL cholesterol or apoA-I **(Table 2).** Conversely, it increased apoA-II and preβ1-HDL **(Table 2)** and decreased HDL apoC-III whereas HDL apoE remained unchanged **(Supplemental Table 4S)**. LCAT and CETP activities did not vary with treatment. FFB decreased the number of medium size HDL particles **(Table 2 and Figure 2)**.

Regarding oxidative parameters, FFB decreased both paraoxonase and PAF-AH activities without significant changes in LOOH and antioxidant capacity **(Table 2)**.

3.3. Changes induced by niacin

ERN/LRP significantly increased HDL cholesterol and apoA-I, and showed a tendency to decrease preβ□-HDL **(Table 2)**. CETP and LCAT mass and activity were decreased after treatment. ERN/LRP did not show any effect on HDL subclass distribution **(Table 2 and Figure 2)**.

Regarding oxidation, PAF-AH and paraoxonase activities were significantly decreased by ERN/LRP **(Table 2).**

3.4. Comparison between treatments

Although ERN/LRP significantly increased HDL cholesterol, the total HDL particle number was not significantly modified by any of the treatments **(Table 2)**.

The HDL spectra of medium and large HDL particles were different between controls and T2DM and were not reversed to normal after any treatment **(Figure 3A)**. No differences in the NMR spectrum of small HDL were observed. **Figure 3B** shows the average HDL particle sizes, confirming that the HDL from T2DM patients is smaller than that of controls, and neither FFB nor ERN/LRP fully correct this alteration. The mean radius for the healthy group (4.7 nm) was higher than that of the T2DM group (4.5 nm) (P=0.002). FFB tended to shift the distribution towards smaller particles, whereas RN/LRP treatment increased the relative concentration of the medium HDL subclass, which consequently, although not significantly, approached the healthy state. The effects of different treatments on the mean HDL radius were significantly different (P=0.042).

4. Discussion

Three essential messages are obtained from our study. The first message is that pharmacological intervention with ERN/LRP and FFB in T2DM patients leads to important HDL particle modifications, ~~in addition to modifications~~ beyond ~~to~~ HDL cholesterol concentrations. The second message is that these composition changes differ according to the HDL cholesterol-increasing medication used. The third message is that neither ERN/LRP nor FFB reverses diabetic HDL alterations *ad integrum*. These differences could, in part, be explained by the mechanisms of action of both medications. FFB is a PPARα agonist that increases proteins associated with lipolysis activity, whereas niacin, among other mechanisms, reduces adipose tissue lipolysis [30, 31]. The post-treatment HDL particles differ depending on the medication used, and they also differ from normal; therefore, the expected effect on cardiovascular risk should be unequal. HDL cholesterol, which is the primary variable that is expected to be modified by ERN/LRP and FFB, increases in different proportions. Although niacin produced a mean 18% increase, FFB did not significantly modify HDL cholesterol. These results are in concordance with previous publications that show similar effects of niacin and FFB [6, 8-10]. Niacin was also the only medication that produced a significant increase in apoA-I. However, consistent with previous observations, only FFB induced a significant 15% increase in apoA-II [32]. Patients with T2DM had significantly higher preβ1-HDL particles, which were further increased by FFB, whereas niacin tended to decrease the levels of preβ1-HDL. The clinical repercussion of this fact is not clear. ~~Although high preβ1-HDL is considered to be a harmful marker,~~ Both high and low levels of preβ1-HDL have been associated with cardiovascular risk and the presence of cardiovascular risk factors [33, 34]. These discrepancies can be explained by different mechanisms, including increased synthesis, decreased maturation,

or both, that can be involved in the origin of preβ1-HDL plasma accumulation. ~~Although increased synthesis leads to an increased capacity for removing cholesterol, the halt in preβ1-HDL maturation would be associated with the contrary. Increased apoA-II has been shown to induce the formation of preβ1-HDL particles that are poor LCAT activators [35-37].~~ ~~Independent of whether these mechanisms are responsible for the changes in preβ1-HDL, our data clearly show that these two agents have a different metabolic impact at this level.~~ Patients with T2DM had higher CETP activity, which tended to be reduced by both treatments, although only after ERN/LRP, this tendency was statistically significant, which suggests that partial inhibition of CETP could be a mechanism that explains the effects of both ERN/LRP and FFB on HDL cholesterol concentrations. T2DM patients had a high LCAT mass, but no significant differences in activity were observed. ERN/LRP significantly reduced LCAT activity in T2DM patients. Despite a different marginal effect on PAF-AH and PON 1 mass and activity, neither fenofibrate nor niacin treatment was associated with better oxidation profile markers according to the lipoperoxide concentration in apoB-lipoprotein-containing depleted plasma, which is an indirect index of HDL oxidation, and the capability of HDL to protect against LDL oxidation~~. The antioxidant protection capability of HDL in T2DM patients is likely influenced by various factors, including their contents of apoA-I and antioxidant enzymes such as PON1 and PAF-AH, as well as the presence of hyperglycaemia and hyperhomocysteinemia [38, 39].~~ Diabetic patients had an HDL fraction with increased proportions of apoA-II and C-III and half the concentration of apoE. Although FFB increased apoA-II, niacin reduced its concentration. FFB reduced apoC-III; however, neither FFB nor ERN/LRP modified the proportion of apoE. The biological impact of these differences is not known (Supplemental Tables 3S and 4S). We speculate that the increased apoA-II after FFB treatment may modify apolipoprotein

exchange between HDL and triglyceride-rich particles, thus decreasing lipoprotein lipase activity and influencing both triglyceride and HDL cholesterol concentrations [35~~][40~~].

The NMR results reinforced that HDL from T2DM patients is clearly different from the healthy group in terms of particle size and number. T2DM patients had fewer HDL particles, and the HDL particles from T2DM patients had smaller radii. These alterations were not reversed by ERN/LRP or FFB.

~~Despite the theoretical benefits of the two studied treatments on the HDL lipid content, the pathological state is not completely corrected.~~

Some limitations of our study are that the intervention period of the study was only 12 weeks; therefore, our results cannot be extrapolated over a longer period of time. The sample size is small due to the comprehensive analyses performed, including metabolomics techniques. This rather small sample size allows only the detection of large effects; however, it does warrant enough power for the main results of the study. The ERN preparation was associated with LRP, so we cannot exclude the lipid effects associated with this product, although if they exist, they seem to be very light.

ERL/LRP has been withdrawn from the market, although other niacin-based pills are available in different countries, and FFB is widely available. The overall conclusions of our work are that neither ERN/LPP nor FFB reverse HDL particle abnormalities associated to T2DM. Moreover these two drugs act differently on HDL. Our results should contribute to a better understanding of the negative results observed in randomized controlled trials using niacin or fenofibrate. In our hands these two drugs don’t improve HDL particle composition, size and metabolism, despite a marginal impact on HDL cholesterol concentrations. Clinicians prescribing these drugs must be aware of their overall impact on HDL particles and lipoprotein metabolism. ~~Although~~

~~each change observed in the HDL fraction of T2DM patients could have a different clinical implication, our aim in this report was to emphasise the important differential effect of these two HDL cholesterol-increasing medications. As previously demonstrated, the HDL cholesterol content is most likely not the best marker of HDL particle characteristics. The development of different tests that target the global HDL particle number, size and function is warranted.~~

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Figure legends

Figure 1. Flow chart of participant enrolment, randomisation and analysis.

Figure 2. Percentage of change between baseline and post-FFB and post-ERN/LRP of HDL lipids, apolipoproteins and enzymes. The data represent the mean ± SD values; n=26. The effect of treatment was analysed using the paired sample Wilcoxon signed rank *test*, **P*<0.05.

Figure 3. HDL particle size distribution in controls and T2DM patients before and after ERN/LRP and FFB treatments. **A)** The coloured curves are the means of the three normalised functions for each group. The area under each curve represents the relative lipid concentration of a particular subclass. B**)** The mean HDL radius in controls and T2DM patients before and after ERN/LRP and FFB treatments.

Table 1. Biochemical characteristics of the control population and T2DM patients

	Control (n=30)	T2DM (n=30)	*P*
Lipoproteins			
Cholesterol (mmol/L)	5.42 ± 0.99	6.14 ± 1.24	**0.030**[#]
Triglycerides (mmol/L)	0.95 (0.73-1.20)	2.39 (1.63-3.49)	**<0.001**
HDL-C (mmol/L)	1.51 ± 0.36	1.05 ± 0.28	**<0.001**
ApoA-I (g/L)	149 ± 15	132 ± 15	**<0.001**
ApoA-II (g/L)	30.3 ± 3.9	28.6 ± 4.8	0.095
ApoB-100 (g/L)	96 ± 22	121 ± 26	**0.001**
preβ1-HDL (µg/ml)	17.8 (11.6-22.6)	22.8 (15.5-27.7)	**0.027**[#]
Enzymes			
CETP mass (µg/ml)	2.5 ± 0.7	2.5 ± 0.7	0.906
CETP activity (pmol/h*µl)	7.2 ± 2.0	9.2 ± 2.9	**0.006**
LCAT mass (µg/ml)	9.6 ± 1.4	10.7 ± 1.9	**0.006**
LCAT activity (FER)	6.28 ± 4.6	11.3 ± 10.6	0.152
PAF-AH (µmol/min*ml)	19.7 ± 5.1	22.7 ± 7.5	0.101
PAF-AH in HDL (µmol/min*ml)	11.9 ± 4.9	12.6 ± 5.8	0.644
Oxidation			
PON1 (mg/L)	78.6 ± 23.9	62.0 ± 23.4	**0.007**
PON3 (mg/L)	1.4 ± 0.5	1.6 ± 0.5	0.255
Lactonase activity (U/L)	6.4 ± 2.9	7.6 ± 2.1	**0.021**[#]
Paraoxonase activity (U/L)	269.2 ± 101.9	320.2 ± 146.4	0.193
LOOH in HDL (µmol/mg)	0.15 ± 0.10	0.19 ± 0.10	**0.044**[#]
Antioxidant capacity (% dienes)	100.1 ± 21.7	91.6 ± 25.9	0.208

HDL subclass particle concentration (μmol/L)			
Large HDL	0.6 (0.4-0.8)	0.5 (0.4-0.7)	0.196
Medium HDL	3.9 (2.9-5.3)	2.9 (1.8-4.2)	**0.004**
Small HDL	7.8 (6.1-9.7)	7.8 (6.2-10.2)	1
Total HDL	13.6 (11.0-15.2)	10.8 (10.0-12.6)	**0.009**

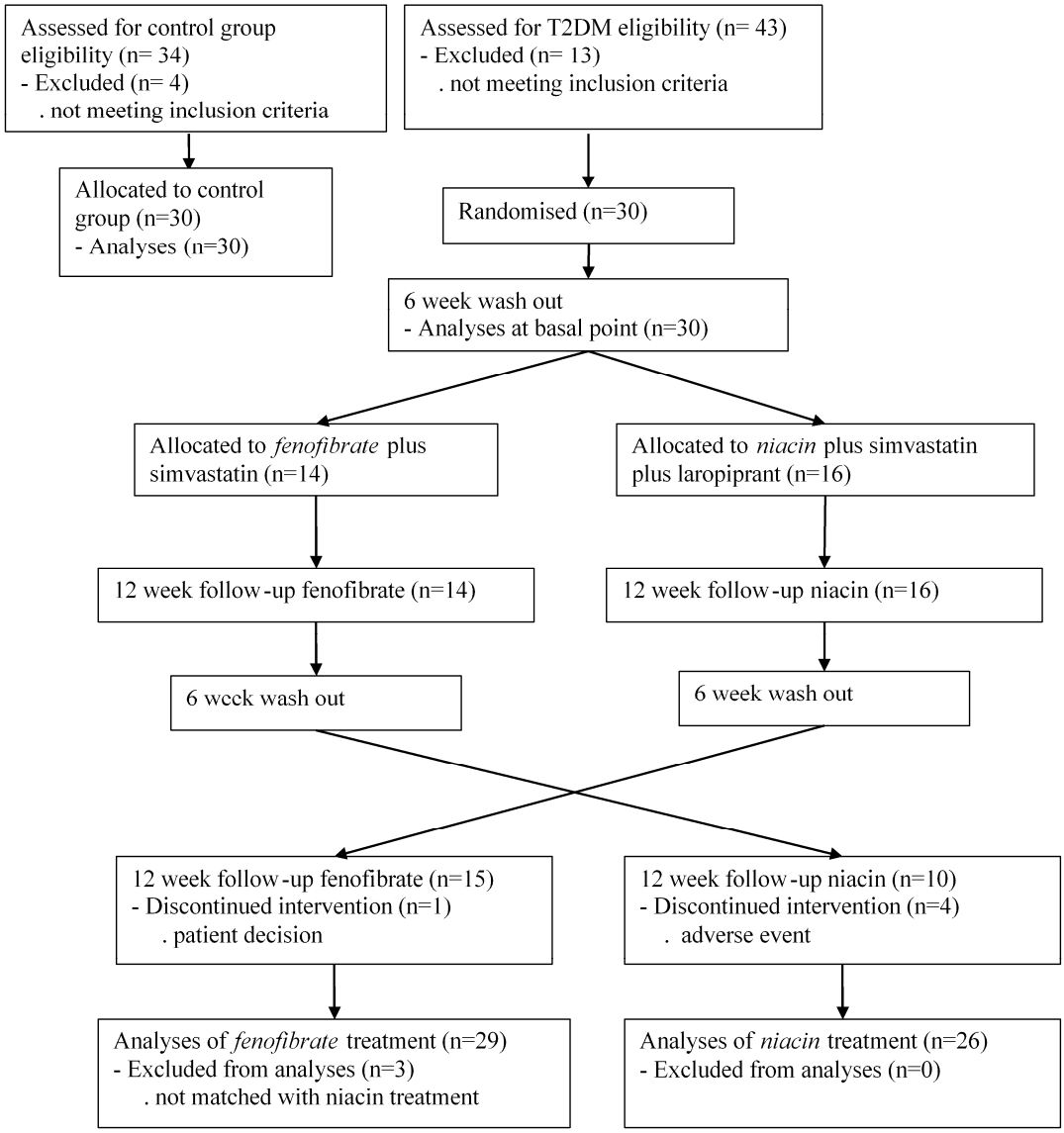
Normal distributed data are shown as the mean ± SD values and non-normal distributed data as the median (interquartile range). The control population and T2DM patients were analysed using the Mann-Whitney U *t*est. [#] Results lost statistical significance at P≤0.05 after we adjusted for α inflation caused by multiple testing.

Table 2. Biochemical characteristics at baseline and post ERN/LPR and FFB treatments in T2DM patients

	T2DM Baseline (n=26)	T2DM Post-FFB (n=26)	*P FFB vs. baseline**	T2DM Post-ERN/LPR (n=26)	*P ERN/LPR vs. baseline**	*P between treatments***
Lipoproteins						
Cholesterol (mmol/L)	6.17 ± 1.27	4.50 ± 0.96	**<0.001**	4.41 ± 0.77	**<0.001**	0.818
Triglycerides (mmol/L)	2.50 (1.98-4.20)	1.86 (1.38-2.62)	**0.006**	1.50 (1.07-2.35)	**0.003**	0.058
HDL-C (mmol/L)	1.03 ± 0.29	1.03 ± 0.28	0.620	1.22 ± 0.36	**0.003**	**0.022**$^{\#}$
ApoA-I (g/L)	1.32 ± 0.16	1.33 ± 0.16	0.284	1.37 ± 0.19	**0.032**	**0.022**$^{\#}$
ApoA-II (g/L)	0.285 ± 0.050	0.327 ± 0.054	**<0.001**	0.285 ± 0.047	0.576	**<0.001**
ApoB-100 (g/L)	1.20 ± 0.27	0.93 ± 0.24	**<0.001**	0.85 ± 0.22	**<0.001**	**0.011**$^{\#}$
preβ1-HDL (µg/ml)	25.5 (16.5-27.9)	28.8 (22.4-37.8)	**0.005**	21.3 (17.7-29.1)	0.603	**0.016**$^{\#}$
Enzymes						
CETP mass (µg/ml)	2.6 ± 0.7	2.1 ± 0.5	**<0.001**	2.1 ± 0.6	**<0.001**	0.435
CETP activity (pmol/h*µl)	9.6 ± 2.9	8.3 ± 2.0	0.080	7.8 ± 2.0	**0.004**	0.065
LCAT mass (µg/ml)	10.6 ± 2.0	9.7 ± 1.8	**<0.001**	9.9 ± 2.3	**0.025**$^{\#}$	0.559
LCAT activity (FER)	11.8 ± 10.8	7.7 ± 8.1	0.180	3.8 ± 5.6	**0.010**$^{\#}$	**0.046**$^{\#}$
PAF-AH (µmol/min*ml)	22.2 ± 7.6	18.1 ± 6.9	**0.006**	17.8 ± 6.7	**0.020**$^{\#}$	0.663
PAF-AH in HDL (µmol/min*ml)	12.2 ± 5.7	10.0 ± 3.5	**0.008**	9.6 ± 4.2	**0.007**	0.463
Oxidation						
PON1 (mg/L)	63.2 ± 24.7	73.7 ± 27.2	**0.015**$^{\#}$	65.5 ± 23.6	0.248	0.060
PON3 (mg/L)	1.6 ± 0.5	1.5 ± 0.7	0.865	1.4 ± 0.5	0.135	0.196
Lactonase activity (U/L)	7.2 ± 1.6	6.2 ± 2.3	0.065	6.8 ± 2.4	0.469	0.339
Paraoxonase activity (U/L)	292.4 ± 114.1	265.1 ± 92.1	**0.023**$^{\#}$	273.9 ± 105.9	**0.020**$^{\#}$	0.716

LOOH in HDL (µmol/mg)	0.20 ± 0.11	0.19 ± 0.10	0.638	0.23 ± 0.12	0.086	**0.046**[#]
Antioxidant capacity (% dienes)	95.0 ± 22.9	87.4 ± 44.6	0.226	96.1 ± 38.4	0.778	0.128
HDL subclass particle concentration (µmol/L)						
Large HDL	0.5 (0.4-0.7)	0.5 (0.2-0.7)	0.909	0.6 (0.4-0.8)	0.114	**0.029**[#]
Medium HDL	3.3 (2.1-4.3)	2.4 (1.9-3.2)	**0.044**[#]	3.3 (2.4-3.9)	0.361	**0.032**[#]
Small HDL	7.8 (6.0-10.0)	8.1 (6.4-9.1)	0.989	7.1 (5.7-8.4)	0.301	0.139
Total HDL	10.8 (9.8-12.5)	10.6 (9.6-13.2)	0.395	10.9 (8.9-13.0)	0.346	0.986

Normal distributed data are shown as the mean ± SD values and non-normal distributed data as the median (interquartile rang). Pre-treatment baseline data were compared with post-treatment data using the paired sample *Wilcoxon* signed rank *test; *P*, the effect of treatment was analysed using the paired sample *Wilcoxon* signed rank *test; **P*. [#] Results lost statistical significance at P≤0.05 after we adjusted for α inflation caused by multiple test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Randomised (n=30)
6 week wash out
- Analyses at basal point (n=30)
Allocated to *fenofibrate* plus simvastatin (n=14)
Allocated to *niacin* plus simvastatin plus laropiprant (n=16)
12 week follow-up fenofibrate (n=14)
12 week follow-up niacin (n=16)
6 week wash out
6 week wash out
12 week follow-up fenofibrate (n=15)
- Discontinued intervention (n=1)
. patient decision
12 week follow-up niacin (n=10)
- Discontinued intervention (n=4)
. adverse event
Analyses of *fenofibrate* treatment (n=29)
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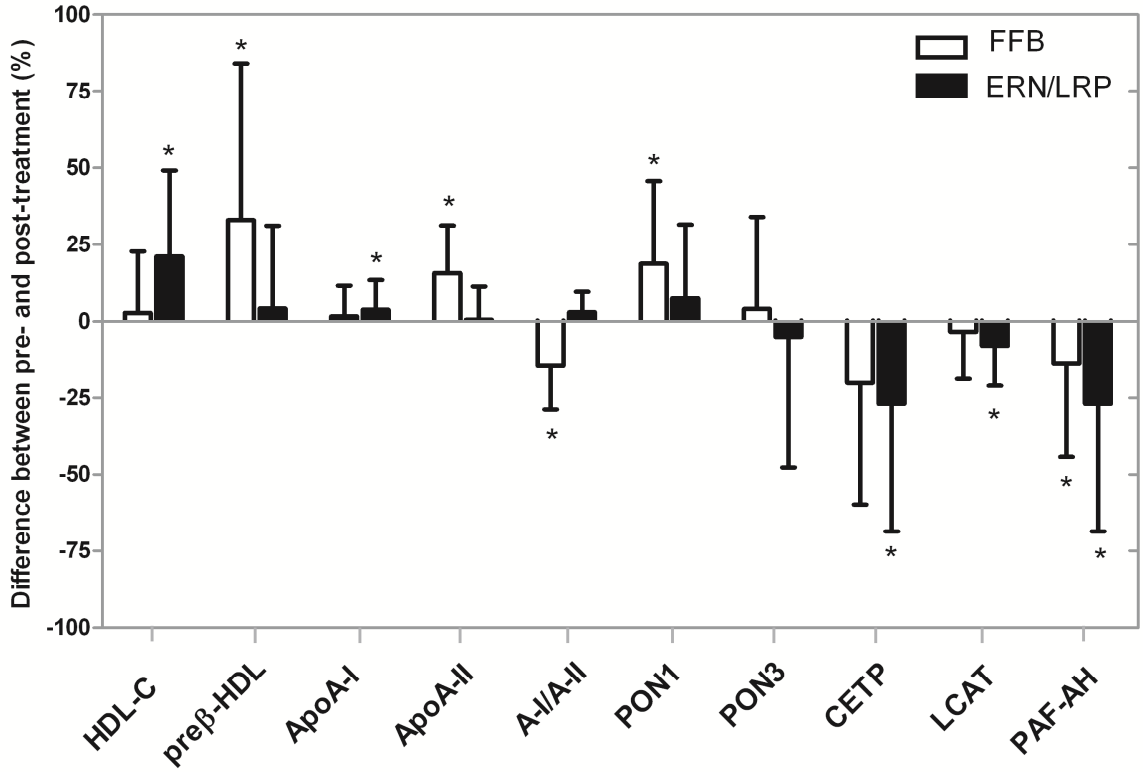

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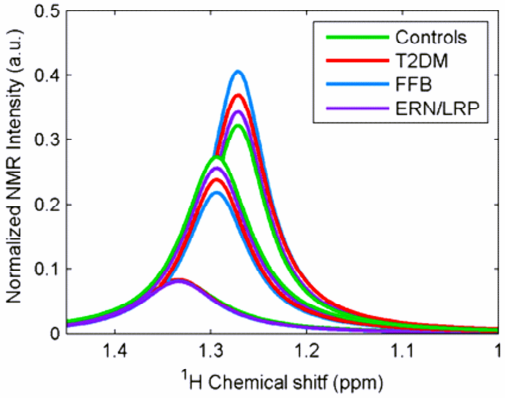

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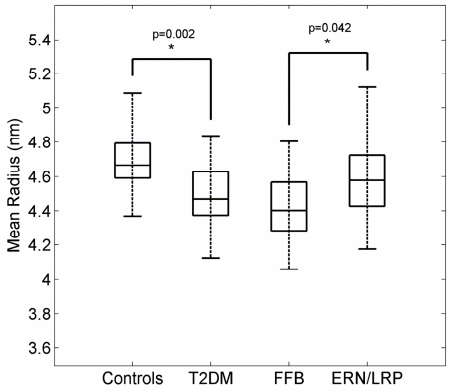


Supplemental material

Methods

1. Enzyme and transfer protein concentrations and activity studies

Lecithin cholesterol acyltransferase (LCAT) and CETP protein concentrations were measured in human serum using a quantitative enzyme-linked immunosorbent assay (ELISA) (American Diagnostica Gmbh). CETP activity was measured using a fluorometric assay (BioVision, USA). LCAT activity was assessed. The cholesterol esterification rate (CER) was measured, and the results were expressed as the fractional cholesterol esterification rate (FER), following the method of Dobiasova [1].

Platelet activated factor acetylhydrolase (PAF-AH) activity was determined using 2-thio-PAF as a substrate (Cayman Chemical, Ann Arbor, MI, USA). This activity was quantified in the serum and HDL after the precipitation of β-lipoproteins using phosphotungstic acid and magnesium ions (Roche Diagnostics).

Lactonase activity was measured in an assay reagent containing 1 mM CaCl2, 0.25 mM TBBL and 0.5 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) in 0.05 mM Tris-HCl buffer, pH = 8.0. The change in absorbance was monitored at 412 nm. The activities were expressed as U/L (1 U = 1 mmol of TBBL hydrolysed per minute). Serum PON1 paraoxonase activity was determined by measuring the rate of hydrolysis of paraoxon at 410 nm and 37ºC in a 0.05 mM glycine buffer, pH 10.5, with 1 mM CaCl2. Activities were expressed as U/L (1 U = 1 μmol of paraoxon hydrolysed per minute).

2. 2D diffusion-ordered ^{1}H NMR spectroscopy (DOSY)

The ucHDL fraction samples were analysed using nuclear magnetic resonance (NMR) spectroscopy according to a modified existing protocol [2]. The ^{1}H NMR spectra were

recorded using a BrukerAvance III spectrometer at 310 K. We used the double stimulated echo (DSTE) pulse program with bipolar gradient pulses and a longitudinal eddy current delay (LED).

3. ucHDL NMR feature extraction

The DSTE methyl signal was fitted with one lorentzian function to obtain the averaged diffusion coefficient (D_{coef}) of the lipoprotein particles. The hydrodynamic radii of the lipoprotein fractions (R_H) were extracted from the Stokes-Einstein equation:

$$R_H = \frac{k_B T}{6\pi\eta D_{coef}} \quad (1)$$

where k_B is the Boltzmann constant, T is the temperature (310 K), and η is the mean viscosity of the solution (0.75 ± 0.02 mPa·s) measured at 310 K using a Cannon-Manning semi-micro capillary viscometer, as described in a previous work [2].

4. HDL particle size distribution and number

The methylene NMR signal had a sufficient resolution to be fitted with three lorentzian functions corresponding to three different HDL subclasses: large, medium and small HDL (approximately 6 nm, 5 nm and 4 nm in radius, respectively). Figure 1S shows the NMR spectrum of large, medium and small HDL subclasses. The methylene group of the lipids, which are primarily esterified cholesterol (EC) and triglycerides (TG), contained in lipoprotein particles resonate at slightly shifted frequencies depending on the size of the particle carrying them; the larger particles resonate at higher frequencies [3]. The area below each lorentzian function is proportional to the concentration of lipids of this particular subclass. The relative concentration of each subclass provides an

estimation of the size distribution of HDL particles. Additionally, to assess the HDL particle number of each subfraction, we transformed the obtained concentrations of EC and TG for each subclass to volume units, as previously reported [4]. Subsequently, we divided the total volume of each subfraction by their associated particle volume, considering lipoproteins as spheres with superficial shells of 2 nm thickness that are composed by phospholipids, proteins and free cholesterol and an internal core of EC and TG, according to the following equation (2):

$$PN_{Subfraction} = \frac{V_{Subfraction}}{\frac{4}{3}.\pi(R_{Subfraction} - Shell\ thickness)^3} \quad \textbf{(2)}$$

Supplemental Tables

Table 1S. Physical and clinical characteristics of the control population and type 2 diabetic patients

	Control (n=30)	Type 2 diabetes (n=30)	*P*
Physical characteristics			
Age (years)	58.1 ± 8.2	58.8 ± 7.6	0.548
Gender (M/F)	19 / 11	19 / 11	1.000
Weigh (kg)	74.3 ± 9.8	85.2 ± 11.8	**0.001**
Waist circumference (cm)	93.1 ± 9.1	103.5 ± 9.5	**<0.001**
BMI (kg/m^2)	26.4 ± 2.5	31.0 ± 3.9	**<0.001**
SBP (mmHg)	126 ± 18	136 ± 16	**0.011**
DBP (mmHg)	76 ± 12	80 ± 9	0.198
Biochemical characteristics			
Homocysteine (µmol/L)	13.3 ± 3.9	13.6 ± 4.5	1.000
Glucose (mmol/L)	5.6 ± 0.6	8.9 ± 2.5	**<0.001**
Fructosamine (µmol/L)	231.4 ± 28.0	283.4 ± 70.2	**0.001**
Creatinine (mg/dl)	0.6 ± 0.2	0.6 ± 0.2	0.211
GOT (U/L)	19 ± 4	24 ± 9	**0.036**
GPT (U/L)	16 ± 6	29 ± 14	**<0.001**
GGT (U/L)	26 ± 25	38 ± 31	**0.008**
Uric acid (mg/dl)	5.1 ± 1.2	6.4 ± 1.8	**0.001**
Bilirubin (mg/dl)	0.7 (0.6-1.0)	0.7 (0.6-1.1)	0.848
Alkaline phosphatase (U/L)	126.6 ± 27.3	162.1 ± 51.4	**0.007**
Urea (mg/dl)	38 ± 7	36 ± 17	**0.077**

Normal distributed data are shown as the mean ± SD values and non-normal distributed data as the median (interquartile range). The control population and type 2 diabetic patients were analysed using the Mann-Whitney U *t*est.

Table 2S. HDL composition of the control population and type 2 diabetic patients

%	Controls (n=30)	Type 2 diabetes (n=30)	*P*
Cholesterol	17.0 ± 1.3	14.4 ± 2.3	**<0.001**
Triglycerides	5.2 ± 1.3	7.6 ± 2.3	**<0.001**
Phospholipids	26.7 ± 2.8	24.9 ± 3.0	0.074
ApoA-I	37.6 ± 2.6	38.8 ± 3.4	0.425
ApoA-II	10.9 ± 1.6	11.9 ± 1.6	**0.042**
ApoC-II	0.5 ± 0.3	0.4 ± 0.3	0.187
ApoC-III	1.7 ± 0.5	2.0 ± 0.5	**0.011**
ApoE	0.4 ± 0.2	0.2 ± 0.1	**<0.001**
Total Protein	51.1 ± 3.0	53.2 ± 3.5	0.062

Normal distributed data are shown as the mean ± SD values. The control population and type 2 diabetic patients were analysed using the Mann-Whitney U *t*est.

Table 3S. Physical and clinical characteristics at baseline and post ERN/LPR and FFB treatments in type 2 diabetic patients

	Type 2 diabetes Baseline (n=26)	Type 2 diabetes Post-FFB (n=26)	*P FFB vs. baseline**	Type 2 diabetes Post-ERN/LPR (n=26)	*P ERN/LPR vs. baseline**	*P between treatments***
Physical characteristics						
Weigh (kg)	86.4 ± 11.9	86.1 ± 12.2	0.260	85.3 ± 12.298	**0.013**	0.196
Waist circumference (cm)	104.6 ± 9.6	105.0 ± 10.1	0.361	104.1 ± 9.9	0.252	**0.014**
BMI (kg/m^2)	31.2 ± 3.7	31.1 ± 4.0	0.316	30.8 ± 4.0	**0.021**	0.212
SBP (mmHg)	136 ± 17	132 ± 14	0.280	131 ± 12	0.221	0.829
DBP (mmHg)	80 ± 9	81 ± 9	0.229	78 ± 10	0.219	**0.009**
Biochemical characteristics						
Homocysteine (µmol/L)	13.9 ± 4.6	15.5 ± 4.0	**0.043**	14.9 ± 4.9	0.112	0.303
Glucose (mmol/L)	8.9 ± 2.7	8.9 ± 1.9	0.501	9.2 ± 2.3	0.148	0.304
Fructosamine(µmol/L)	283.2 ± 60.6	286.5 ± 71.3	0.213	337.6 ± 110.4	**0.002**	**0.013**
Creatinine(mg/dl)	0.6 ± 0.2	0.6 ± 0.2	**0.011**	0.6 ± 0.2	0.709	**0.032**
GOT (U/L)	26 ± 9	25 ± 7	0.628	24 ± 8	0.231	0.276
GPT (U/L)	30 ± 14	29 ± 17	0.294	27 ± 15	0.058	0.091
GGT (U/L)	40 ± 32	32 ± 26	**0.001**	30 ± 18	**0.007**	0.931
Uric acid (mg/dl)	6.5 ± 1.8	5.1 ± 1.2	**<0.001**	6.9 ± 1.9	0.454	**<0.001**
Bilirubin (mg/dl)	0.7 (0.6-1.1)	0.6 (0.5-0.8)	**0.001**	0.7 (0.5-0.8)	**0.009**	0.542
Alkaline phosphatase (U/L)	166.6 ± 53.2	133.8 ± 42.6	**<0.001**	164.3 ± 53.2	0.929	**0.001**
Urea (mg/dl)	35 ± 17	40 ± 9	**0.001**	36 ± 17	0.275	**0.042**

Normal distributed data are shown as the mean ± SD values and non-normal distributed data as the median (interquartile range). Pre-treatment baseline data were compared with post-treatment data using the paired sample *Wilcoxon* signed rank *test; *P*, the effect of treatment was analysed using the paired sample *Wilcoxon* signed rank *test; **P*.

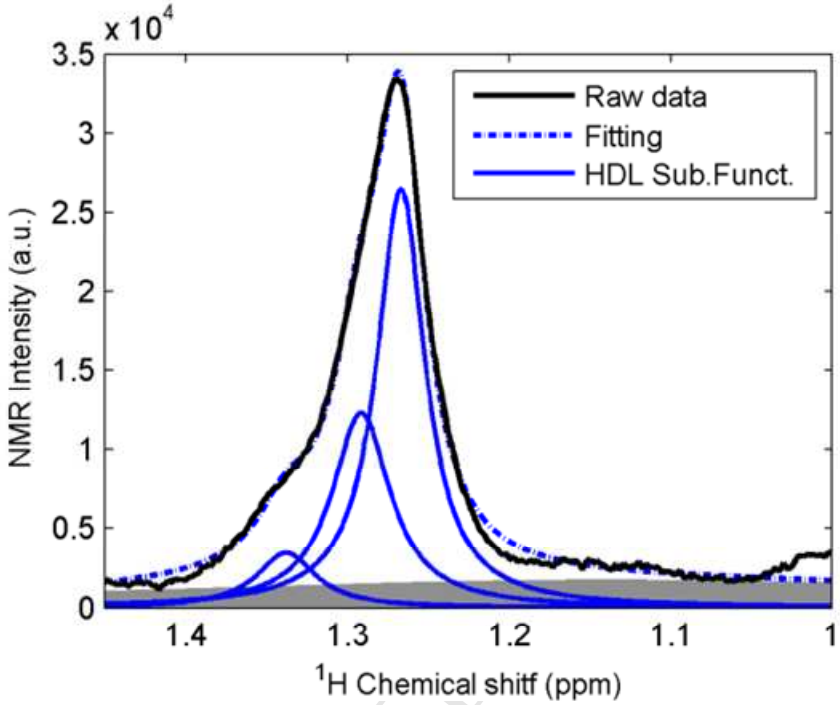
Table 4S. HDL composition at baseline and post ERN/LPR and FFB treatments in type 2 diabetic patients

%	Type 2 diabetes Baseline (n=26)	Type 2 diabetes Post-FFB (n=26)	*P FFB vs. baseline**	Type 2 diabetes Post-ERN/LPR (n=26)	*P ERN/LPR vs. baseline**	*P between treatments***
Cholesterol	14.0 ± 2.2	14.1 ± 2.6	0.647	15.1 ± 2.8	**0.015**	**0.032**
Triglycerides	7.9 ± 2.3	7.2 ± 2.1	0.125	7.1 ± 2.5	0.238	0.611
Phospholipids	25.0 ± 2.9	24.2 ± 3.1	0.388	23.9 ± 3.4	0.253	0.572
ApoA-I	38.6 ± 3.3	39.0 ± 3.5	0.703	40.1 ± 4.0	0.121	0.080
ApoA-II	11.9 ± 1.6	13.3 ± 2.2	**<0.001**	11.2 ± 2.6	**0.036**	**<0.0001**
ApoC-II	0.4 ± 0.3	0.4 ± 0.3	0.642	0.5 ± 0.4	0.583	0.092
ApoC-III	2.0 ± 0.6	1.7 ± 0.6	**0.023**	2.0 ± 1.0	0.186	0.117
ApoE	0.2 ± 0.1	0.2 ± 0.1	0.316	0.1 ± 0.1	0.861	0.091
Total Protein	54.5 ± 3.3	53.9 ± 3.1	0.170	53.9 ± 3.1	0.253	0.258

Normal distributed data are shown as the mean ± SD values. Pre-treatment baseline data were compared with post-treatment data using the paired sample *Wilcoxon* signed rank *test; *P*, the effect of treatment was analysed using the paired sample *Wilcoxon* signed rank *test; **P*.

Supplemental Figure

Figure 1S. The NMR spectrum is fitted with three functions that correspond to three different HDL subclasses: the left, central and right curves (blue solid curves) correspond to the large (~6 nm), medium (~5 nm) and small (~4 nm) HDL subclasses, respectively. The blue dashed curve corresponds to the sum of the three functions and reproduces the NMR signal. An auxiliary curve, represented by a grey zone, is used to optimise the fitting and corresponds to the base line signal.



References

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- Diabetic patients have an important alteration of HDL composition and metabolism.
- Fenofibrate and niacin impact differently on HDL composition and metabolism.
- The HDL particles after fenofibrate and niacin treatment are different among them.
- None of the two drugs reverse type 2 diabetic patients HDL to normal state.
- HDL quality can explain the low clinical effectiveness of both drugs.