TREATMENT WITH FIBRATES IS ASSOCIATED WITH HIGHER LAL ACTIVITY IN DYSLIPIDEMIC PATIENTS

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ABSTRACT

Lysosomal acid lipase (LAL) is responsible for the hydrolysis of cholesteryl esters (CE) and triglycerides (TG) within the lysosomes; generated cholesterol and free fatty acids (FFA) are released in the cytosol where they can regulate their own synthesis and metabolism. When LAL is not active, as in case of genetic mutations, CE and TG accumulate in the lysosomal compartment, while the lack of release of cholesterol and FFA in the cytosol leads to an upregulation of their synthesis. Thus, LAL plays a central role in the intracellular homeostasis of lipids. Since there are no indications about the effect of different lipid-lowering agents on LAL activity, aim of the study was to address the relationship between LAL activity and the type of lipid-lowering therapy in a cohort of dyslipidemic patients.

LAL activity was measured on dried blood spot from 120 patients with hypercholesterolemia or mixed dyslipidemia and was negatively correlated to LDL-cholesterol levels. Among enrolled patients, ninety-one were taking one or more lipid-lowering drugs, as statins, fibrates, ezetimibe and omega-3 polyunsaturated fatty acids. When patients were stratified according to the type of lipid-lowering treatment, i.e. untreated, taking statins or taking fibrates, LAL activity was significantly higher in those with fibrates, even after adjustment for sex, age, BMI, lipid parameters, liver function, metabolic syndrome, diabetes and statin use. In a subset of patients tested after 3 months of treatment with micronized fenofibrate, LAL activity raised by 21%; the increase was negatively correlated with baseline LAL activity.

Thus, the use of fibrates is independently associated with higher LAL activity in dyslipidemic patients, suggesting that the positive effects of PPAR-α activation on cellular and systemic lipid homeostasis can also include an improved LAL activity.

Keywords: Lysosomal acid lipase, fibrates, statins, dyslipidemia.

1. Introduction

Lysosomal acid lipase (LAL) is coded by the *LIPA* gene on chromosome 10 and it is responsible for the hydrolysis of cholesteryl esters (CE) and triglycerides (TG) within the lysosomes [1]. Generally, these lipids have been internalized by the receptor-mediated endocytosis of apoB-containing lipoproteins, but can also derive from intracellular lipid droplets through the activation of the autophagic process [2]. The reaction catalyzed by LAL generates unesterified cholesterol (UC) and free fatty acids (FFA), which are released into the cytosol where they can regulate their own synthesis and metabolism [1]. When LAL is not active, as in case of mutations in the *LIPA* gene affecting protein synthesis or function, CE and TG accumulate in the lysosomal compartment in the peculiar form of microvesicles, especially in the liver and in macrophages throughout the body. Mutations in the *LIPA* gene cause two recessive diseases depending on residual LAL activity, namely Wolman Disease (WD) and Cholesteryl Ester Storage Disease (CESD) [3]. WD is the neonatal-onset and fulminant type, while CESD can be diagnosed in childhood or adulthood. Clinically, CESD patients present with hepatomegaly, splenomegaly, malabsorption and increased cardiovascular risk [4]. Biochemically, hypercholesterolemia (variably associated with hypertriglyceridemia) and low plasma levels of HDL-cholesterol have been described, together with elevation of liver enzymes [4]. The alteration of the lipid profile is the consequence of the lack of release of UC and FFA in the cytosol of LAL-deficient hepatocytes; indeed, the activation of the sterol regulatory element-binding proteins (SREBPs) leads to an upregulation of cholesterol and FFA synthesis and of VLDL secretion. In addition, the lower generation of oxysterols from UC in the cytosol results in an impaired activation of liver X receptors (LXRs), with a consequent reduction of ABCA1 expression and HDL biogenesis [5]. Thus, the impaired intracellular lipid metabolism in LAL-deficient cells can

affect circulating lipid levels. However, while single nucleotide polymorphisms within the *LIPA* gene were associated with the risk of coronary artery disease [6], it is not clear whether LAL activity is associated with plasma lipid levels in the general population.

Besides the recent availability of recombinant LAL for enzyme replacement therapy, CESD patients are usually given statins to manage the hypercholesterolemia, while low HDL-cholesterol and hypertriglyceridemia could suggest the use of fibrates [7,8]. These molecules can modulate plasma lipid levels by rewiring cellular lipid metabolism; however, their direct effect on LAL activity has not been addressed to date. Thus, aim of the present study was to investigate the relationship between LAL activity, biochemical/anthropometric variables and the type of lipid-modifying therapy in a cohort of dyslipidemic patients.

2. Methods

2.1 Patients

Patients with hypercholesterolemia or mixed dyslipidemia were enrolled among those attending the Lipid Clinics at the Niguarda, Policlinico and Bassini Hospitals, as part of a study aimed at the identification of candidates for genetic LAL deficiency. Patients were selected on the base of the following criteria: (i) total cholesterol ≥ 250 mg/dl or LDL-cholesterol ≥ 160 mg/dl without lipid-lowering therapy, (ii) body mass index (BMI) ≤ 28 kg/m^2 [9]. The study conformed to the guidelines set out in the Declaration of Helsinki and was approved by the pertinent IRBs; all enrolled patients gave written informed consent for participation in the study. None of the enrolled patients was affected by genetic LAL deficiency. The database was retrospectively analyzed to assess the relationship between LAL activity and biochemical/clinical features at enrollment.

2.2 Clinical evaluation

Body mass index (BMI), waist circumference (WC), concomitant diseases and medications were recorded at the time of LAL evaluation. The presence of hepatomegaly was assessed by liver examination and/or on the base of abdominal imaging (abdominal ultrasound, computerized tomography or magnetic resonance), revealing fatty liver appearance or hepatomegaly. Fatty liver index (FLI) was calculated as described [10]. In all subjects, daily alcohol intake was lower than 20 g in females and 30 g in males (confirmed by at least one family member).

2.3 Biochemical analyses

Blood samples were collected in EDTA tubes after an overnight fast. Dried blood spot cards (DBS, GE Healthcare Whatman 903) were immediately prepared, dried overnight at room temperature and stored at -20°C until assayed for LAL activity. Plasma samples were obtained by low-speed centrifugation and stored at 4°C.

Plasma levels of liver enzymes, total and HDL cholesterol, triglycerides and glucose were determined by enzymatic techniques on a Roche c311 automatic analyzer (Roche Diagnostics). LDL-cholesterol was calculated by the Friedewald's formula.

LAL activity on DBS was measured by fluorescence using 4-methylumbelliferone palmitate (4-MUP, Cayman Chemicals), cardiolipin (Avanti Polar Lipids) and the selective LAL inhibitor Lalistat 2 (Sigma Aldrich), according to the method of Hamilton et al. [11]. Briefly, a 3.2 mm spot was punched from DBS card and eluted in 200 μl H_2O for 1h at room temperature. Forty μl of eluted sample were incubated with H_2O or with 30 μM Lalistat-2 for 10 minutes at 37°C and then with 150 μl of 0.15 M acetate buffer pH 4.0, 1% Triton X-100 containing cardiolipin and 4-MUP for 3h at 37°C. Assay was performed in 96-well black plates. The generation of fluorescent 4-methylumbelliferone (4-MU) was detected by the Synergy H1 Multi-Mode microplate reader and GEN5 software (BioTek); excitation was set at 320nm and emission at 460nm. A standard curve of 0–2.5 nmol 4-MU (Sigma Aldrich) was built. LAL activity was calculated by subtracting the activity in the inhibited reaction (with Lalistat 2) from uninhibited reaction (with H_2O) and expressed as nmol of generated 4-MU/spot/h. The coefficient of variation of the assay is 8.5%. Normal values of LAL activity are >0.80 nmol/spot/h. DBS cards were tested for quality by measuring beta-galactosidase activity as described [12]; new DBS cards were prepared if beta-galactosidase activity was below 90 pmol/spot/h [12].

In a subgroup of 11 patients taking no medications and with clinical indication for fibrates after dietary intervention (i.e. plasma levels of triglycerides above 200 mg/dl), DBS cards were collected before and after 3 months of treatment with micronized fenofibrate 145 mg/day.

2.4 Statistical analysis

Continuous variables are expressed as mean±SD, and categorical variables as cases and percentages, if not otherwise stated. Homogeneity of variance was assessed using a Levene's test and normal distribution was tested by Shapiro-Wilk test. Non-normally distributed variables were log-transformed before proceeding to the analysis. Comparisons between groups of treatment were assessed by one-way ANOVA for independent samples. When variables were still non-normally distributed after log-transformation, a Kruskal-Wallis test was performed. Differences in LAL activity between treatment groups were assessed by covariance analysis (ANCOVA) and adjusted for age, sex, BMI, total cholesterol, triglycerides, LDL-cholesterol, HDL-cholesterol, presence of hepatomegaly, concomitant diseases and medications. A Spearman's rank-order correlation was run to assess the relationship between LAL activity and all other variables. Differences between pre- and post-treatment with micronized fenofibrate were assessed by paired t-test or Wilcoxon signed-rank test for normally and non-normally distributed variables, respectively. Pearson's product-moment correlation was used to assess the relation between percent change and baseline LAL activity. All tests were 2-sided and P values < 0.05 were considered as statistically significant. Statistical analysis was performed by using SPSS version 25.0 software (SPSS Inc., Chicago, USA).

3. Results

3.1 Features of enrolled patients

Dyslipidemic patients were mainly males and average BMI was within the normal range (Table 1). Overall, they displayed a moderate mixed dyslipidemia, with elevation of both total cholesterol and triglyceride levels (Table 1). Fasting glucose, liver function and LAL activity were in the normal range (Table 1). Eight subjects (6.7%) were in secondary prevention, 10 (8.3%) were diabetics and 36 (30%) were hypertensive. Nineteen patients (15.8%) had metabolic syndrome according to the NCEP/ATPIII criteria [13]. LAL activity was negatively correlated with LDL-cholesterol levels ($r_S(115)=-0.217$, $P=0.020$). No other correlations were found between LAL activity and the biochemical/anthropometric variables listed in table 1. Ninety-one patients (75.8%) were taking one or more lipid-lowering drugs on a stable treatment regimen for at least 4 weeks (Table 1). Among the 57 patients treated with statins, only 6 were given a high-intensity one. Thirty patients were taking statins alone, 13 statins plus ezetimibe (2 with fenofibrate and 3 with 1 g/die omega-3 polyunsaturated fatty acids - PUFAs), 7 statins plus 1 g/die omega-3 PUFAs and 7 statins plus fibrates (1 with omega-3 PUFAs). Among the 36 patients treated with fibrates, 21 were given fenofibrate 145 mg, 8 bezafibrate 400 mg and 7 gemfibrozil 600-900 mg. Eighteen were given fibrates alone, 8 fibrates plus 1-3 g/die omega-3 PUFAs and 1 fibrates plus ezetimibe. Seven patients were treated with 1-3 g/die omega-3 PUFAs alone.

Patients were then divided according to the type of lipid-lowering treatment as indicated in table 2 and mean LAL activity was calculated for each group. Due to similar mean LAL levels, three main treatment categories were identified: patients untreated or taking only omega-3 PUFAs (category 1), patients taking statins with or without ezetimibe or omega-3 PUFAs

(category 2) and patients taking fibrates with or without statins or omega-3 PUFAs (category 3).

3.2 Relationship between LAL activity and treatment category

Features of patients belonging to the 3 different treatment categories are reported in table 1. Patients were comparable for sex distribution, WC, total cholesterol, and liver function. Dyslipidemic patients taking statins displayed lower BMI and TG values, and higher HDL-cholesterol if compared to the other two groups. Fasting glucose was slightly increased in the fibrate group. Patients taking no medications were younger and their plasma levels of LDL-cholesterol were higher (Table 1). While the prevalence of hypertension and cardiovascular events were comparable between the 3 categories, a higher rate of metabolic syndrome and diabetes where found among fibrate-treated patients compared to the other groups (19% and 64% respectively, vs 7% and 6% in statin-treated group, and 6% and 0% in the untreated group, $P<0.001$ for metabolic syndrome and $P=0.003$ for diabetes).

Interestingly, LAL activity was significantly different between the 3 groups, with patients taking fibrates showing the highest activity (Table 1 and Figure 1). LAL activity was significantly different between the treatment groups also when adjusted for sex, age, BMI, total cholesterol, triglycerides, LDL-cholesterol, HDL-cholesterol presence of hepatomegaly, presence of metabolic syndrome or diabetes and statin use (Table 1).

3.3 Effect of fibrates on LAL activity

To further address the effects of fibrate treatment on LAL activity, a pilot study was performed. Eleven male patients (mean age 43.3±8.7 years) were selected among those taking no medications and with TG values above 200 mg/dl after dietary intervention. They were given 145 mg of micronized fenofibrate for 3 months without other concomitant lifestyle

196 changes. After treatment, patients showed no changes of BMI, LDL-cholesterol and
197 transaminases. Although with great variability, total cholesterol and TG were reduced by 8.7%
198 and 32.1%, respectively, while HDL-cholesterol increased by 18.9% (Table 3). Fatty liver
199 index also tended to decrease. DBS were collected before and after treatment with fenofibrate
200 for LAL activity assay. Even in this small number of patients, a significant 20.8% increase of
201 LAL was observed after fenofibrate (P<0.001, Figure 2). Interestingly, LAL increased in 10
202 patients (range 6.7-51.3%), while was almost unchanged in 1 patient (+0.7%). Furthermore,
203 the extent of LAL increase was inversely related to baseline LAL (r=-0.664, P=0.026, Figure
204 2).

4. Discussion

LAL activity was evaluated in a cohort of dyslipidemic patients with a low incidence of other metabolic alterations; indeed, body weight, blood glucose and parameters of liver function were in the normal range. The first finding of the study is the negative correlation between LAL activity and plasma LDL-cholesterol in absence of a genetic defect, suggesting that intracellular LAL may play a role in the modulation of systemic cholesterol levels even in the normal range of activity. Consistently, several genome wide association studies found an association between single nucleotide polymorphisms in the *LIPA* gene and the incidence of coronary artery disease, although the impact of such variants on LAL expression and activity is still debated [6,14-17]. The second aim of the study was to investigate the effect of lipid-lowering drugs on LAL activity, which was unaddressed to date. We showed for the first time that the treatment with fibrates is associated with higher LAL values in dyslipidemic patients, while omega-3 PUFAs and statins showed no effect. The association was significant even after adjustment for several anthropometric, clinical and biochemical parameters. In a recent paper, Baratta et al. showed that statin use was less frequent in NAFLD patients having a LAL activity below the median of their cohort [18]. We did not observed a significant effect of statin treatment on LAL activity, but this discrepancy could be due to the striking differences between the patients enrolled in the two studies. Indeed, Baratta et al. analyzed obese patients (mean BMI was 30.5 mg/k^2) with fatty liver and higher incidence of metabolic syndrome (70.5%) if compared to our patients.

To further investigate the effect of fibrates on LAL activity, a pilot study was performed. Eleven patients with clinical indication for fibrates were given micronized fenofibrate for 3 months and LAL activity was measured before and after treatment. Standing all the limitations of this small and uncontrolled study, we were able to show for the first time that LAL activity

significantly improved after fenofibrate and that the lower was baseline LAL the higher was the improvement mediated by fenofibrate. In addition, although far from statistical significance, a tendency towards a reduction of fatty liver index was also detected after fenofibrate. These findings suggest that the activation of PPAR-α receptors by fibrates could rewire intracellular lipid metabolism also leading to an increase of LAL activity and are worth of a confirmation in larger and controlled studies with novel and more potent PPARα agonists, as elafibranor or pemafibrate [19,20].

As stated above, the relevance of our study is limited by the small sample size of both the retrospective and intervention studies, and by the uncontrolled design of the pilot study with fenofibrate. However, it provides additional evidence on the usefulness of fibrates in the management of dyslipidemias. Atherogenic dyslipidemia, characterized by small dense LDL, elevated triglycerides and reduced HDL-cholesterol [21], which can benefit from fibrates, is frequently associated with insulin resistance and with other features of the metabolic syndrome; fibrates were shown to improve glycemic control in these patients [22]. Our results suggest a potential additional effect of fibrates through the improvement of LAL activity, especially when it is impaired (as in patients with fatty liver, a common feature of the metabolic syndrome [18]).

Dedicated studies are needed to address the mechanisms responsible for LAL increase after PPARα activation. However, based on the known effects of PPARα agonists, some speculations are possible. First, PPARα agonists could increase LAL expression through the activation of transcription factor EB (TFEB). TFEB is the master regulator of lysosomal biogenesis and, consequently, of LAL expression. Recently, peroxisome proliferator responsive elements were identified in the promoter region of TFEB and it has been shown that PPARα, together with PPAR gamma coactivator 1α and retinoid X receptors, can

promote the transcriptional activation of TFEB [23]. In addition, PPARα agonists could directly promote LAL activity through their modulation of intracellular lipid metabolism. Unesterified cholesterol and free fatty acids are the product of the LAL reaction in the lysosomes; while UC is actively transported in the cytosol by the Niemann-Pick disease type C1 protein, FFA likely diffuse between the two compartments according to the concentration gradient [24]. Since PPARα activation increase the catabolism of intracellular FFA through the stimulation of the β-oxidation [25], it could improve LAL activity by promoting the flux of generated FFA to the cytosol.

In conclusion, the activation of the PPARα receptors could positively affect systemic lipid homeostasis also by increasing LAL activity, through the stimulation of intracellular catabolism of lipoproteins and lipid droplets.

Declarations of interest:

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The work has not been published previously, except in the form of a meeting abstract (C. Pavanello *et al*. Atherosclerosis supplements 32 (2018) 152). Its publication is approved by all authors and tacitly by the responsible authorities where the work was carried out.

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Table 1. Characteristics of patients divided according to treatment category

	All patients	Category 1	Category 2	Category 3	*P* values
Main treatment		None	Statins	Fibrates	
N	120	36	48	36	-
Age, y	57.8 ±13.8	50.3±14.3	61.2±13.4	60.8±10.7	0.001
Sex, m/f	90/30	28/8	33/15	29/7	0.419
BMI, kg/m^2	25.6±2.9	25.8±2.3	24.8±3.0	26.5±3.1	0.015
WC, cm	96.3±6.6	94.4±6.3	96.6±7.0	97.9±6.2	0.165
Total cholesterol, mg/dl	226.1±46.2	238.8±40.3	216.5±43.6	226.3±52.6	0.053
Triglycerides, mg/dl	196.2±129.6	214.9±136.0	157.7±62.7	227.8±171.4	0.025
LDL-cholesterol, mg/dl	140.7±41.7	156.1±41.8	134.2±44.2	134.3±34.7	0.040
HDL-cholesterol, mg/dl	47.3±12.9	42.9±12.6	52.1±11.1	45.1±13.5	0.002
Fasting glucose, mg/dl	93.1±27.2	86.5±10.1	86.6±14.8	107.4±41.5	0.001
AST, U/l	25.0±7.4	25.6±8.1	23.1±6.4	27.1±7.4	0.042
ALT, U/l	30.4±15.2	34.5±18.8	26.0±10.1	32.6±16.2	0.086
Gamma-GT, U/l	38.5±43.4	49.8±65.0	33.0±35.2	36.4±28.2	0.650
Hepatomegaly, n (%)	40 (34.0)	11 (32.3)	12 (25)	17 (47.2)	0.101
Fatty liver index	46.9±26.9	56.2±24.0	48.2±19.1	59.1±19.1	0.093
LAL activity, nmol/spot/h	1.21±0.42	1.09±0.40	1.17±0.31	1.37±0.53	0.018
Adjusted LAL activity, nmol/spot/h	n.a.	1.03 (0.80-1.25)	1.23 (1.01-1.45)	1.37 (1.20-1.55)	0.019

Data are mean±SD or as number (percentage) of cases. Adjusted LAL activity is expressed as means (95% CI). Differences between treatment categories were tested by one-way ANOVA or Kruskal-Wallis, where appropriate. For categorical variables, Pearson's Chi-square test was used. In the adjusted model, LAL activity was adjusted for age, sex, BMI, total

cholesterol, triglycerides, LDL-cholesterol, HDL-cholesterol, glucose, hepatomegaly, statin use, metabolic syndrome and diabetes.

Table 2. LAL activity according to lipid-lowering treatment

Treatment type	n	LAL activity (nmol/spot/h)	Treatment category	*P*-value
None	29	1.09±0.42	1	
Omega-3 PUFAs	7	1.06±0.37	1	0.874*
Statins	30	1.20±0.33	2	
Statins+ezetimibe	11	1.14±0.30	2	
Statins+omega-3 PUFAs	7	1.12±0.31	2	0.573[#]
Fibrates	19	1.42±0.50	3	
Fibrates+statins	9	1.43±0.66	3	
Fibrates+omega-3 PUFAs	8	1.30±0.61	3	0.691[#]

Data are expressed as mean±SD. *P*-values were calculated by one-way ANOVA for treatment category 1 (*) and by Kruskal-Wallis test for treatment category 2 and 3 ([#]).

Table 3. Biochemical and anthropometric parameters before and after treatment with fenofibrate

	Before	After	*P* values
BMI, kg/m^2	27.3±2.5	27.5±2.5	0.945
Total cholesterol, mg/dl	238.9±41.3	218.0±51.8	0.077
Triglycerides, mg/dl	294.1±138.8	199.7±118.3	0.111
LDL-cholesterol, mg/dl	146.0±49.7	130.1±42.6	0.171
HDL-cholesterol, mg/dl	40.2±10.7	47.8±18.8	0.073
AST, U/l	26.3±13.4	31.3±10.8	0.174
ALT, U/l	33.9±24.4	44.8±21.3	0.164
Gamma-GT, U/l	34.7±17.6	39.2±32.2	1.000
Fatty liver index	69.7±17.7	62.0±20.7	0.093
LAL activity, nmol/spot/h	1.03±0.31	1.24±0.30	<0.001

Data are expressed as mean±SD. Differences between before and after treatment with micronized fenofibrate were assessed by Wilcoxon signed-rank test or Wilcoxon-rank, where appropriate.

FIGURE LEGENDS

Fig. 1. LAL activity in dyslipidemic patients according to treatment category.
LAL activity was measured on DBS. Boxes indicate the median and 25^{th}-75^{th} percentiles, capped bars the 10^{th}-90^{th} percentiles. None: patients untreated or given omega-3 PUFAs, n=36. Statins: patients taking statins alone or in combination with ezetimibe/omega-3 PUFAs, n=48. Fibrates: patients taking fibrates alone or in combinations with statins/omega-3 PUFAs, n=36.

Fig. 2. LAL activity before and after treatment with fenofibrate
Panel A, LAL activity was measured on DBS from 11 patients before and after 3 months treatment with 145 mg micronized fenofibrate. Panel B, correlation between baseline LAL values and percent change after treatment.