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Lysosomal acid lipase activity in children with dyslipidemia and hepatic dysfunction

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Abstract

Background: Lysosomal acid lipase (LAL) enzyme, is responsible for the hydrolysis of intracellular triacylglycerol and cholesterol esters. We investigate the LAL activity (LAL-A) in patients with hepatic dysfunction and/or dyslipidemia and determine the associated clinical and biochemical parameters.

Methods: This prospective, cross-sectional study included 360 children (3 months -18 years; 40 control, and 320 screening patients). Demographic data, major clinical and laboratory findings, LAL-A and possible biomarkers were evaluated. Screening group was divided into two: LAL-A<0.6 nmol/ml/h (Group1); LAL-A ≥ 0.6 nmol/ml/h (Group 2). LAL-A predictive model was evaluated using logistic regression.

Results: The mean LAL-A in the screening group (1.43 ± 2.05 (0.03-16.8) nmol/ml/h) was significantly reduced compare to controls ($p < 0.001$). No LAL deficiency was detected. There was a negative correlation between LAL-A and low-density lipoprotein cholesterol, triglyceride, and alanine aminotransferase (ALT) levels. LAL-A in patients with chronic fatigue ($p = 0.002$), hepatomegaly ($p = 0.013$) and splenomegaly ($p = 0.001$) were significantly lower compare to those without. The median thiobarbituric acid reactive substances, myeloperoxidase, chitotriosidase, hs-CRP, Citokeratin levels in Group 1 were higher compare to the controls ($p < 0.005$).

Conclusions: LAL-A was reduced in paediatric patients with dyslipidemia and/or elevated transaminase. Our final multivariable predictive model for reduced LAL-A included: ALT, triglyceride, and hepatomegaly.

Abbreviations: ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; BMI: Body Mass Index; CESD: Cholesteryl Ester Storage Disease; CI: Confidence Interval; HDL-C: Low-Density Lipoprotein Cholesterol; hsCRP: High-Sensitivity C-Reactive Protein; LAL: Lysosomal Acid Lipase; LAL-A: LAL activity; LDL-C: High-Density Lipoprotein Cholesterol; MPO: Myeloperoxidase; OR: Odds Ratio; R: Reduced; SR: Significantly Reduced; TBARS: Thiobarbituric Acid Reactive Substances.

Introduction

Pediatric lysosomal acid lipase (LAL) deficiency caused by LIPA gene mutations is associated with two phenotypes Wolman's disease and Cholesteryl ester storage disease (CESD). Deficiency in LAL activity (LAL-A) (< 1% in Wolman's disease, 1-12% of the normal in CESD) leads to impaired hydrolysis of intracellular triacylglycerol and cholesterol esters [1-3].

Clinically, LAL deficiency is manifested by hepatosplenomegaly, hepatosteatosis, hepatic insufficiency, elevations in transaminases, total cholesterol, low-density lipoprotein cholesterol (LDL) and triglyceride levels, decreased in high-density lipoprotein cholesterol (HDL-C) and atherosclerosis [4]. So, lipid metabolism - dyslipidemia [1,5,6] and hepatic involvement [7] are hot topics in the in investigation of children with LAL deficiency.

In this study, the aim was to perform a selective LAL-A screening in pediatric patients with hepatic dysfunction and/or dyslipidemia, and investigate the clinical and laboratory biomarkers chitotriosidase (used in the diagnosis and follow-up of lysosomal diseases), cytokeratin-18 (plays a role in apoptosis in non-alcoholic steatohepatitis), hsCRP (a valuable indicator in atherosclerosis and non-alcoholic steatohepatitis), thiobarbituric acid reactive substances (TBARS) and myeloperoxidase (MPO) (important roles in lipid peroxidation) which might be connected with LAL activity.

Material and methods

Subject sampling

Patients (3 months -18 years old) with unexplained liver dysfunction (presented with at least one of the following finding: persistent elevation in ALT and/or AST, hepatomegaly, hepatic steatosis) and/or dyslipidemia (presented with at least one of the following finding: high LDL-C (≥ 160 mg /dl), low HDL-C (≤ 35 mg/dl)) were included. Patients with obesity, liver disease of known etiology and genetically confirmed familial hyperlipidemia (LDLR, APOB and PCSK9) were excluded from the study. Consent forms were obtained from the children and their parents. The study was approved by Local Ethics Committee.

Data collection

Height and weight measurements were made at the admission. Body mass index (BMI) for each patient was calculated as weight

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(kg) divided by height (meters) squared. Lipid profile, hematological, biochemical parameters and abdominal USG findings were evaluated. LAL-A and sphingomyelinase (reference enzyme) were measured from all patients, whereas chitotriosidase, hsCRP, cytokeratin-18, MPO and TBARS were evaluated only from patients with reduced LAL-A.

Methods

LAL-A was measured fluorometrically using 4-methylumbelliferyl palmitate substrates in dry blood sample [8]. Samples were measured within two weeks and LAL activity was reported after two measurements. Screening group was divided into two groups: LAL-A<0.6 nmol/ml/h (Group1); LAL-A ≥ 0.6 nmol/ml/h (Group 2). Chitotriosidase enzyme activity was measured after the incubation of patient serum at 37°C for about 3 hours, fluorescence formed by the addition of ethylenediamine solution and was read as fluorometric excitation: 360 nm, emission: 445 nm [9]. Patients with very low chitotriosidase activity that might be genetic deficient were excluded from the study. Serum hsCRP levels were measured using the CRP HS ELISA kit (DRG Diagnostics-EIA-3954) based on monoclonal antibody. All serum samples were analyzed in duplicate at the same time with the ELISA kit (Cloud-Clone Corp. SEB231Hu, for cytokeratin 18-human) in which KRT-18 specific antibody coated plates was used for measurement of Cytokeratin-18. For measurement of myeloperoxidase activity, the serum sample was diluted to 1/20 with phosphate buffer. After centrifugation, the supernatant was separated. Hexadecyl trimethyl ammonium bromide was added onto 50 microliters of serum homogenate and then the sample was frozen 3 times at -80 °C. Then o-dianisidine was added on to 30 microliters of each sample and absorbance values were recorded for 10 minutes at 460 nm. TBARS was measured after addition of 1 mL of thiobarbituric acid solution to 0.5 mL of serum. After 20 minutes boiling of 100ºC and centrifuging at 2000 rpm for 10 minutes, colorimetric measurement was made at 532 nm wavelength in the supernatant.

Statistical analysis

All analyzes were performed using SPSS 17.0 statistical package program. The relationship between categorical variables was tested by Chi-square test and the relationship between numerical variables was analyzed by Spearman Correlation analysis. Mann Whitney U Test was used for comparison of two independent median values. Univariable associations with a reduced LAL activity were evaluated using logistic regression models and summarized with odds ratios (OR) and 95% confidence intervals (CIs). A multivariable model was developed using stepwise selection, with the *P* value for a feature to enter or leave the model set to 0.05.

Results

360 children aged 3 months -18 years were included in the study. In the selection of the cases, the presence of at least one of dyslipidemia, hepatosteatosis, hepatomegaly or transaminase elevation was requested (N: 320). Healthy children were nominated as control group (N: 40) and they were matched for age and gender with the screening group.

The median age of all subjects was 5.5 (3 months -18) years. Consanguineous marriage in the screening group was significantly high 115 (31%) ($p < 0.05$). Familial hyperlipidemia was observed in 130 (36%) patients. Characteristics of screening patients and control group were presented at table 1. Weight, height, hemoglobin, platelet, parameters, HDL-C, urea, creatinine, albumin were significantly low, whereas LDL-C, Triglyceride, AST, ALT, total bilirubin were significantly high in the screening group ($p < 0.05$).

Table 1. Demographic and laboratory characteristics of the investigated patients

	Control (n=40)	**Screening (n=320)**
Age (months-year)	7 (3 months-17years)	4 (3 months-18years)
Gender *(n,* %)		
Girl	23 (%57.5)	183 (%57.2)
Boy	17 (%42.5)	137 (%42.8)
Weight SDS*	0.225 ((-1.92)-1.58)	-0.76 ((-5.7)-2.48)
Height SDS*	0.135 ((-1.88)-1.81)	-0.81 ((-5.4)-2.2)
Hb*(g/dl)**	12.7 (10.3-15.3)	11.7 (6.7-16.3)
MCV*(f/L)*	81 (66-89)	79.1 (67-106)
Leukocytes (10 ^ 3 / µL)	8520 (4370-15900)	8590 (1760-32400)
Platelet (10 ^ 3 / µL) *	326500 (156000-597000)	302000 (35000-1097000)
INR	0.92 (0.72-1.1)	0.98 (0.6-2.4)
Total Cholesterol (mg / dl)	150.5 (115-175)	146 (54-340)
LDL Cholesterol (mg / dl) *	85 (55-109)	93 (12-249)
HDL Cholesterol (mg / dl) *	48 (36-70)	26 (3-44)
Triglyceride (mg / dl) *	81 (27-120)	135 (13-2180)
ALT (U / L) *	16 (8-30)	51 (3-1761)
AST (U / L) *	28 (15-37)	44 (12-1860)
Glucose (mg / dl)	84 (61-118)	87 (26-146)
Total Bilirubin (mg / dl) *	0.42 (0.08-1.2)	0.54 (0.09-23)
Direct Bilirubin (mg / dl)	0.09 (0-0.66)	0.10 (0.01-15)
Urea (mg / dl) *	23.5 (12-42)	21 (4-76)
Creatinine (mg / dl) *	0.5 (0.27-1)	0.3 (0.1-4.8)
Albumin (g / dl) *	4.3 (3.2-5)	3.7 (1.6-5.2)

*: Statistically significant difference between the two groups ($p < 0.05$); ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; HDL: High-Density Lipoprotein Cholesterol ; INR: International Normalized Ratio; LDL: Low-Density Lipoprotein Cholesterol; Hb: *Hemoglobin*; MCV: Mean Corpuscular Volume

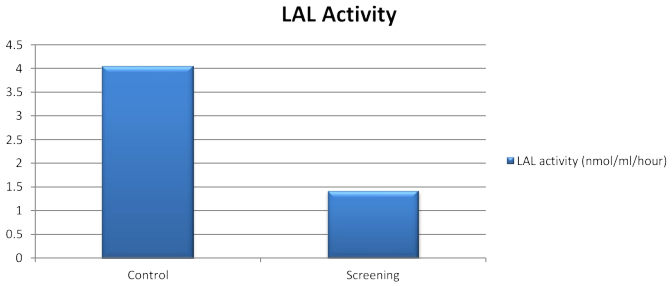

Figure 1. LAL activity of control and screening groups

The mean LAL-A in the screening group was 1.43 ± 2.05(0.03-16.8) nmol/ml/h; significantly lower comparing to the healthy controls ($p < 0.001$). No LAL deficiency (LAL<0.03 nmol/ml/h) was detected (Figure 1).

To investigate the relationship between LAL-A and other clinical and laboratory biomarkers, patients were divided into two groups: Group 1 (LAL-A<0.6 nmol/ml/h (significantly reduced) and Group 2 (LAL-A ≥ 0.6 nmol/ml/h (reduced). The reference interval was defined as 0.26-6.2 nmol/ml/hour. LAL-A below the tenth percentile (0.6 nmol/ml/h) was accepted as a cut-off for the enzyme sufficiency.

The clinical and laboratory characteristics of patients from Group 1 compared to the Group 2 were shown in the presence of splenomegaly lower the mean LAL-A to 0.36 nmol/ml/h (0.03-0.49) ($p=0.001$). Total cholesterol levels were higher ($p = 0.033$), and HDL-C was lower in Group 1 ($p = 0.017$) (Table 2).

Assessment of the possible clinical factors affecting LAL-A was performed by analyzing each parameter: age, gender, growth retardation, chronic fatigue symptoms, hepatomegaly, jaundice, splenomegaly,

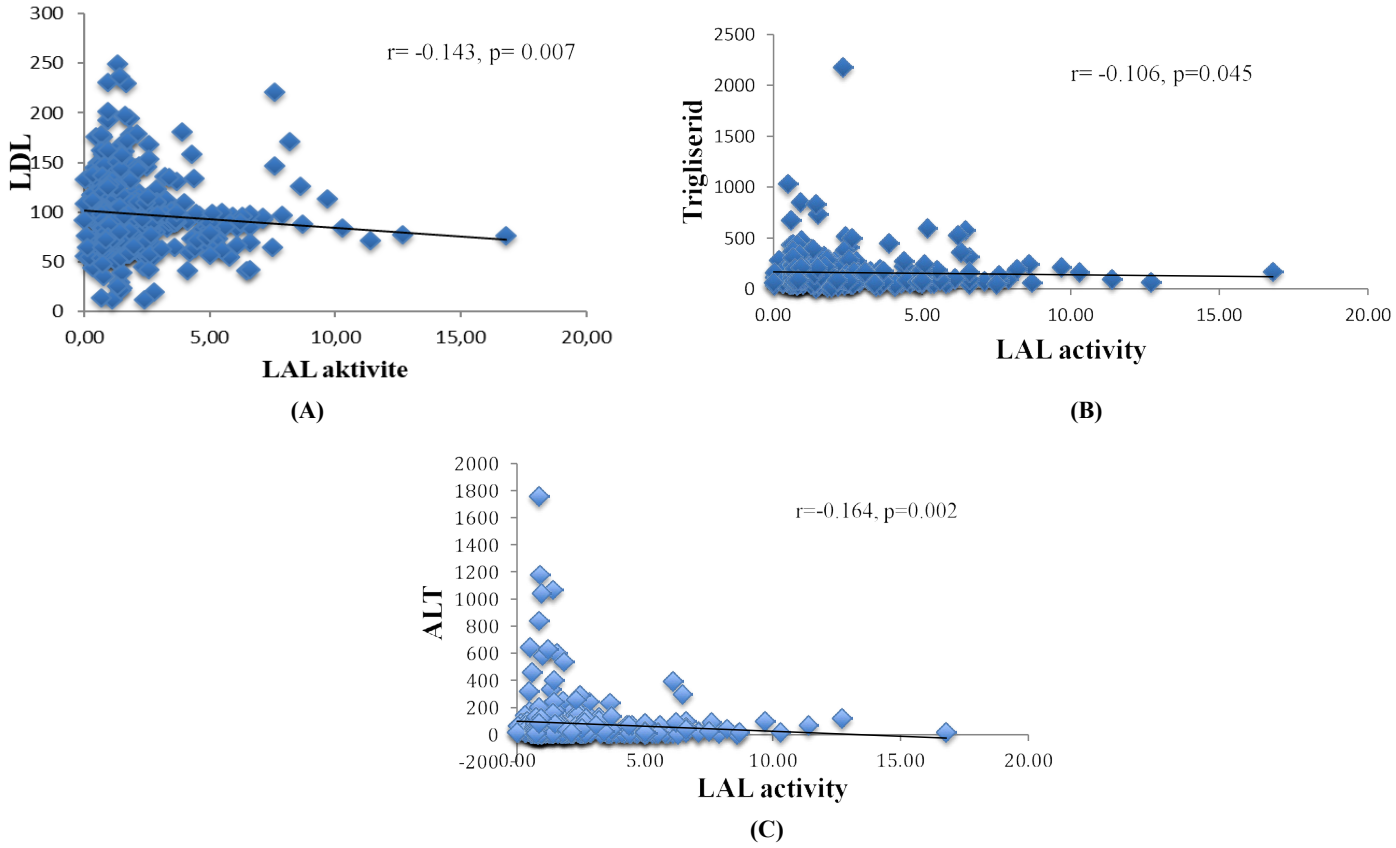

Figure 2. Relationship between LAL activity and LDL-cholesterol levels (A), triglyceride levels (B), ALT levels (C).

Table 2. Symptoms and clinical findings based on LAL level in the screening group

	Group 1 (N=45) (significantly reduced LAL-A/ *(n, % / min-max)*	**Group 2 (N=275) (reduced LAL-A/ *(n, % / min-max)***
Abdominal distension	10 (%22.2)	36 (%13.1)
Abdominal pain	8 (%17.8)	36 (%13.1)
Chronic fatigue	11 (%24.4)	67 (%24.4)
Diarrhea	1 (%2.2)	9 (%3.3)
Growth retardation	7 (%15.6)	76 (%27.6)
Hepatomegaly	15 (%33.3)	71 (%25.8)
Splenomegaly *	13 (%28.9)	45 (%16.4)
Hypertension	10 (%22.2)	34 (%12.4)
Jaundice	6 (%13.3)	22 (%8)
Total Cholesterol (mg / dl) *	156 (101-250)	144 (54-340)
LDL -Cholesterol (mg / dl)	108 (44-176)	91 (12-249)
HDL- Cholesterol (mg / dl) *	27 (7-44)	26 (3-44)
Triglyceride (mg / dl)	151 (38-1034)	134 (13-2180)
ALT (U / L)	65 (10-648)	48 (3-1761)
AST (U / L)	43 (16-1860)	44 (12-989)
GGT (U / L)	15 (5-389)	17 (3-586)
ALP (U / L)	160 (20-869)	169 (34-2790)

*: Statistically significant difference between the two groups ($p < 0.05$). GGT: g-glutamyl transpeptidase; ALP: Alkaline Phosphatase; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase

USG findings. Patients with chronic fatigue have the lowest LAL-A (0.46 (0.03-0.59) nmol/ml/h), significantly different ($p = 0.002$) from those without fatigue (1.80 (0.66-16.8)) nmol/ml/h. LAL-A in patients with accompanying hepatomegaly was 0.48 (0.03-0.59) nmol/ml/h), significantly lower ($p = 0.013$) to those without hepatomegaly (1.79 (0.66-12.79 nmol/ml/h). Although the mean LAL-A (1.02 (0.03-10.3) nmol/ml/h) of 22 (7%) in patients with hepatosteatosis on USG was lower than those with no hepatosteatosis (1.44 (0.04-16.8) nmol/ml/h), the difference was not statistically significant ($p = 0.094$). LAL-A were negatively correlated with LDL-C, triglyceride and ALT (Figure 2).

Independent predictors of significantly reduced LAL activity in univariable predictive model included: chronic fatigue, hepatomegaly, splenomegaly, LDL-C, HDL-C, Triglyceride, AST and ALT levels. Our multivariable predictive model included: ALT, Triglyceride levels and hepatomegaly (Table 3).

The comparison of the group with significantly reduced LAL-A (Group 1) with the healthy control group in terms of oxidative stress and possible biomarker was presented in table 4.The median TBARS 14.38 (5-37.5) mmol/ml, Chitotriosidase 12.85 (2.59-500.67) mmol/ml/h, hs-CRP 3.33 (0.03-42.81) mg/L, Citokeratin (CK-18) 26.92 (2.58-52.8) ng/ml levels in Group 1 were higher compare to the controls ($p < 0.005$). MPO (1.12 (0.12-30.91)) (U/ml) levels were higher, too ($p < 0.001$) (table 4).

Discussion

The main outcome of our study was the fact that pediatric patients with liver dysfunction (persistent elevation in ALT and/or AST, hepatomegaly, hepatic steatosis) and/or dyslipidemia (high LDL-C (≥ 160 mg /dl), low HDL-C (≤ 35 mg/dl)) may have a reduced LAL-A. The significantly reduced LAL-A was found in 14% of them. The other patients have a reduced, but sufficient enzyme levels. Nobody was enzyme deficient. It was reported that the enzyme activity between 0.15 - 0.40 nmol/punch/hour could be related with LIPA gene heterozygous carriers [10]. But on the other hand, it was underlined

Table 3. Univariable and multivariable predictive model for reduced LAL activity

	Univariable predictive model			Multivariable predictive model		
	OR	95 %CI	P	OR	95 %CI	P
Chronic fatigue	0.68	0.52-0.89	0.006			
Hepatomegaly	1.18	1.09-1.27	0.000	1.28	1.10-1.48	0.001
Splenomegaly	0.32	0.16-0.65	0.002			
LDL-Cholesterol ≥ 160 mg/dl	0.238	0.031-1.802	0.001			
HDL- Cholesterol < 35 mg/dl	0.163	0.056-0.474	0.005			
Trigliserid ≥ 150 mg/dl	1.455	0.773-2.736	0.000	2.28	1.73-2.68	0.001
ALT ≥ 60 u/l	1.819	0.964-3.434	0.000	1.18	1.08-1.43	0.001
AST ≥ 60 u/l	0.947	0.486-1.847	0.540			

ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; HDL: High-Density Lipoprotein Cholesterol; LDL: Low-Density Lipoprotein Cholesterol; OR: Odds Ratio

Table 4. Comparison of possible biomarkers measured in Group 1 and healthy control group

	Control (n=40)	Grup1(LAL<0.6) (n=45)
TBARS *(mmol/ml)**	11.25 (3.75-29.38)	14.38 (5-37.5)
MPO *(U/ml)***	0.53 (0.26-10.78)	1.12 (0.12-30.91)
Chitotriosidase *(mmol/ml/h)**	12.85 (2.59-50.67)	14.57 (3.73-77.7)
hs-CRP *(mg/L)**	2.56 (0.13-13.09)	3.33 (0.03-42.81)
Citokeratin(CK-18) *(ng/ml)**	21.04 (2.2-34.93)	26.92 (2.58-52.8)

* Statistically significant difference between the two groups (p <0.05). ** Statistically significant difference between the two groups (p<0.001). TBARS: Thiobarbituric Acid Reactive Substances; MPO: Myeloperoxidase; hsCRP: High-Sensitivity C-Reactive Protein.

that it was not reliable approach to identify the carriers [11]. The fact that LAL activity was previously reported to be reduced in patients with severe liver disease, nonalcoholic fatty liver disease [12-17] brought us to idea to clarified the possible model of conditions related with significantly reduced LAL-A. Chronic fatigue, hepatomegaly, splenomegaly, elevated LDL-C, triglyceride, AST and ALT levels were determined as the most important clinical and laboratory factors associated with reduced LAL-A. Some of them were independently reported by different investigators [12-17]. For example, Shteyer et al. [12] reported no correlation between ALT and LAL, but Baratta et al. [15] found the negative one. On the other hand, our final multivariable predictive model included: only ALT, Triglyceride levels and hepatomegaly as factors which presence coexistence is predictable for significantly reduced LAL-A. Keep in mind that clinical course of CESD is widely variable this model will provide simple criteria for the first step of the screening.

The interesting and important finding of our study was the fact that triglyceride level demonstrated diagnostic predominance compare to the other lipid's components. The reason of these findings is probably hidden in the pathogenesis of the disease. Recent research demonstrated that the inability to hydrolyze triglycerides due to decreased LAL activity results in storage of triglycerides in liver lysosomes and increased triglyceride levels in blood. Then the decrease of intracellular free cholesterol leads to the 'up' regulation of HMG-CoA reductase which can stimulate cholesterol synthesis; in addition, it also causes increases in VLDL cholesterol and LDL-C levels and this explains the dyslipidemia picture in LAL deficiency [18,19].

High oxidative stress and macrophage activated parameters demonstrated in our study and reported previously in the literature [1] supported the hypothesis about increased oxidative status in patients with significantly reduced LAL-A. The differences between Group 1 and healthy controls were evaluated to test the hypothesis that lipid peroxidation might be increased in cases with significantly reduced LAL-A. The higher levels of TBARS, MPO, Chitotriosidase, hs-CRP and Citokeratin supported this hypothesis. Individually some of these biomarkers were reported by different researchers. Bernstein et al, stated that chitotriosidase can be used for the diagnosis and treatment follow-up in CESD, based on their review of 135 CESD clinical and laboratory findings [1]. It is well known that measurement of circulating CK-18 levels can be used as a noninvasive biomarker to determine the degree of steatohepatitis in non-alcoholic fatty liver disease. In the study of Gonzalez et al. [20] CK-18 levels were found to be increased in all liver diseases (fatty liver, alcoholic liver disease, chronic viral hepatitis, autoimmune hepatitis, cholestasis, transplantation, and hepatocellular carcinoma).

Limitations

The most important limitation of this study was the lack of genetic analysis of cases with significantly reduced LAL-A. Another milder limitation was small number of healthy control subjects.

Despite these limitations, to the best of our knowledge, there are no data in the literature evaluating possible predictive models for reduced LAL-A based on the data from broad spectrum and large pediatric population including dyslipidemic and hepatic features of CESD.

Conclusion

This study supports the concept that dyslipidemic pediatric patients and those with elevated transaminases have a reduced LAL-A. It provides a proof of concept about mean LAL-A (1.43 ± 2.05 (0.03-16.8) nmol/ml/h). In spite of the fact that no patients of LAL deficiency were identified, it supplies data about LAL significantly reduced LAL-A in that population (LAL activity < 0.6 nmol/ml/h in 14% of the patients). On the other hand, the study clearly demonstrated that reduced LAL-A was correlated with biomarker like TBARS, MPO, chitotriosidase, hsCRP and CK-18. The main output of our study is creation of the multivariable predictive model for significantly reduced LAL-A which include: ALT, Triglyceride levels and hepatomegaly.

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Ethical approval

This study was approved by Ege University Medical Faculty Clinical Trials Ethical Committee (13-11/88)

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