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Short Communication

Platelet count may impact on lysosomal acid lipase activity determination in dried blood spot

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ABSTRACT

Background: We aimed to evaluate the influence of white blood cell (WBC) and platelet (PLT) counts on dried blood spot (DBS)-determined lysosomal acid lipase (LAL) activity in a large group of healthy subjects.

Methods: One-hundred-and-seventy-two healthy subjects aged ≥18 were enrolled. Complete clinical biochemistry and LAL activity in DBS were determined. In 35 subjects, WBCs and PLTs were isolated, and LAL activity was measured in both blood cell populations. Univariate and multivariate analyses to DBS-LAL activity were performed.

Results: Mean age of subjects was 44.8 ± 17.2 years, 43.6% were males, and mean DBS-LAL activity was normal (1.0 ± 0.3 nmol/spot/h). LAL activity in WBCs was significantly higher than in PLTs (458.9 ± 133.6 *vs* 235.0 ± 88.3 nmol/mg/h, $p < 0.001$). However, LAL activity in DBS correlated more strongly with that in PLTs ($r = 0.65$, $p < 0.001$) than with that in WBCs ($r = 0.49$, $p < 0.01$). Consistently, in the multivariate model, DBS-LAL activity was independently associated only with PLT count ($\beta = 0.39$, $p < 0.001$).

Conclusions: PLT number may impact on the result of the DBS-LAL test, and a consideration of PLT count is recommended before interpreting LAL activity in DBS.

1. Introduction

Lysosomal acid lipase deficiency (LAL-d) is an autosomal recessive disease characterized by a complete or almost complete absence of LAL activity, which produces two well-defined lysosomal storage disorders, *i.e.* Wolman disease (WD) and cholesterol ester storage disease (CESD) [1]. The strong hepatic phenotype of LAL-d, essentially consisting of massive microvesicular steatosis evolving to cirrhosis and liver failure, has recently opened lines of investigation aimed to verify if LAL activity is impaired also in the contest of non-alcoholic fatty liver disease (NAFLD). Indeed, thanks to the availability of a new test on dried blood spot (DBS) [2], which has significantly simplified the determination of LAL activity, LAL function has been investigated in cohorts of patients with chronic liver disease [3–5]. LAL activity was found to be reduced in adult patients with NAFLD [3], and inversely associated with hepatic fibrosis in paediatric NAFLD [4]. Lastly, we found LAL function to be significantly reduced in patients with liver cirrhosis of different etiology [5].

At the same time that this evidence points to the possible contribution of impaired LAL function to the pathophysiology of liver damage, some considerations concerning the LAL test performed in DBS should be raised. Indeed, in comparison to classical assays in white blood cells (WBCs) or fibroblasts [6], in which lysosomal enzyme activity is normalized for total protein concentration, in the DBS test the enzyme activity is not normalized for proteins, and it is expressed as nmol/punch/h [2]. Since most of lysosomal activities tested in DBS are of leukocyte origin, and the number of WBCs in a blood spot depends on the WBC count/mm^3, conditions characterized by leukopenia, such as advanced liver disease, can be expected to affect DBS-determined lysosomal enzyme activity. Indeed, in patients with liver cirrhosis, we observed that DBS-determined LAL activity was independently associated with WBC count [5]. Interestingly, in this same study, we found that LAL activity was even more strongly associated with platelet (PLT) count, both in cirrhotic patients and in healthy controls, thus suggesting that thrombocytopenia should be considered as another possible

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confounder of LAL activity determination in DBS. PLTs are known to contain lysosomes [7], however the contribution of PLTs to DBS-determined lysosomal enzyme activities has never been reported.

Based on this background, we aimed to evaluate the influence of WBC and PLT counts on DBS-determined LAL activity in a large group of healthy subjects. Moreover, to go deeper on this issue, we also isolated WBCs and PLTs of a subgroup of these subjects, and analyzed the correlation between LAL activity in DBS and that measured in WBCs and in PLTs.

2. Patients and methods

Healthy subjects aged ≥18 undergoing general medical assessments or preoperative evaluation for orthopedic surgery at the University Hospital Campus Bio-Medico of Rome between January 2015 and December 2015 were enrolled. Subjects with known medical history or blood tests suggestive of dyslipidemia, diabetes mellitus, liver, and heart or kidney disease, were excluded. Epidemiological, anthropometric, clinical and biochemical data were recorded. All subjects agreed to participate to the study signing an informed consent and the protocol was approved by the Ethics Committee of the University Campus Bio-Medico of Rome.

All subjects underwent blood drawing after a 12-hour fast and blood was spotted onto Whatman paper #903 (within 13 mm diameter circles) on the day of venipuncture and allowed to dry overnight at room temperature. Samples were stored double-bagged with desiccant at −20 °C and analyzed within 1 week of collection. In a subgroup of 35 subjects, fraction-specific LAL activity was determined in WBCs and PLTs. WBCs and PLTs were isolated from 10 ml of blood collected into Vacutainer EDTA and into Vacutainer Sodium Citrate, respectively. PLT separation was carried out by centrifugation at 200 ×*g* at room temperature for 15 min and the PLT-rich plasma (PRP) was centrifuged again for 10 min. After, Anticoagulant Citrate Dextrose Solution (ACD-A) (39 mM citric acid, 75 mM sodium citrate, 135 mM dextrose pH 7.4) was added to supernatant in a ratio 1:10. PLTs were pelleted by centrifugation at 1600 ×*g* for 3 min, and stored at −20 °C until processed. For the isolation of WBCs, 10% dextran was added. Erythrocytes were allowed to sediment at room temperature for 45 min and the upper phase centrifuged at 1125 ×*g* for 10 min. The pellet was washed with 0.9% NaCl and stored at −20 °C until the analysis. Protein content was determined by the BCA protein assay (Pierce).

LAL activity was determined after eluting a 3.2 mm circular punch from the DBS using the inhibitor Lalistat-2, as previously described [5]. In WBCs and PLTs, LAL activity was measured based on the method of Civallero et al., with some modifications [8]. Reactions were performed in duplicate.

Data are expressed as mean (standard deviation) or number and percentage, as appropriate. Comparisons between groups were performed by the χ^2 or Fisher-exact test for categorical variables and by the Mann-Whitney test for continuous numeric variables. Linear regression analysis was carried out to evaluate the association of different variables with DBS-determined LAL activity. Variables whose association with LAL activity at the univariate analysis showed a $p < 0.10$ entered the multivariate model. A $p < 0.05$ was considered statistically significant. R 3.2.3 software for Mac (R Foundation) was used for statistical analyses.

3. Results and discussion

One-hundred-and-seventy-two healthy subjects were recruited. Mean age was 44.8 ± 17.2 years and 43.6% of them were male. Mean LAL activity was in the normal range (1.1 ± 0.4 nmol/spot/h), and, although it was mildly reduced (<0.8 nmol/spot/h) in 41 subjects (23.8%), no cases in the range of heterozygous (<0.4 nmol/spot/h) or homozygous (<0.15 nmol/spot/h) carriers of LIPA gene mutations were found [2]. Also mean WBC and PLT counts were in the normal range (6.100 ± 1.700 and 241.000 ± 60.200 cells/mm^3, respectively).

Univariate analyses to DBS-determined LAL activity disclosed significant and near-significant direct associations with PLT and WBC counts, respectively, and near-significant inverse associations with age and male sex (Table 1). In the multivariate model, DBS-determined LAL activity was independently associated only with PLT count ($\beta = 0.39$, $p < 0.001$).

When LAL activity was measured in isolated fractions of WBCs and PLTs obtained from a fixed aliquot (10 ml) of whole blood of the 35 subjects, it was significantly higher in the WBC- than in the PLT-fraction (458.9 ± 133.6 *vs* 235.0 ± 88.3 nmol/mg/h, $p < 0.001$), thus suggesting that in a blood drop the main contribution for the LAL enzymatic activity originate from WBCs rather than from PLTs. Conversely, LAL activity in DBS correlated with that determined in PLTs ($r = 0.65$, $p < 0.001$) more strongly than with that determined in WBCs ($r = 0.49$, $p < 0.01$) (Fig. 1). Altogether, these results are the first to demonstrate that PLTs significantly contribute to LAL activity assayed in DBS, and that PLT count is strongly and independently associated with DBS-determined LAL function.

Differently from determination in WBCs or fibroblasts, where LAL activity is normalized for the protein content, the number of cells containing lysosomes in the blood spot, *i.e.*, WBCs and PLTs, could impact on the result of the DBS test. Actually, the problem of a reduced WBC count has been already faced when determining in DBS the activity of other lysosomal enzymes [9,10]. Recently, we reported that DBS-determined LAL activity was independently associated with WBC count in

Table 1
Univariate and multivariate linear regression analysis for LAL activity in DBS (10^{-2} nmol/spot/h) in the total population.

	Univariate					Multivariate				
Variables	Estimate	β	95%CI		p	Estimate	β	95%CI		p
Age (years)	−0.30	−0.14	−0.62	0.03	0.07	−0.36	−0.17	−0.67	−0.06	0.02
Sex (male)	−9.90	−0.13	−21.12	1.32	0.08	−4.67	−0.06	−15.21	5.87	0.38
BMI (kg/m^2)	−0.37	−0.05	−1.53	0.79	0.53	–	–	–	–	–
Hypertension	−0.88	−0.01	−16.55	14.80	0.91	–	–	–	–	–
Glycaemia (mg/dL)	0.09	0.02	−0.51	0.70	0.76	–	–	–	–	–
Total cholesterol (mg/dL)	−0.09	−0.08	−0.27	0.10	0.37	–	–	–	–	–
HDL cholesterol (mg/dL)	0.09	0.04	−0.30	0.49	0.64	–	–	–	–	–
LDL cholesterol (mg/dl)	−0.06	−0.05	−0.27	0.14	0.54	–	–	–	–	–
Triglycerides (mg/dL)	−0.05	−0.07	−0.18	0.07	0.41	–	–	–	–	–
AST (U/L)	−0.21	−0.06	−0.82	0.41	0.51	–	–	–	–	–
ALT (U/L)	−0.38	−0.12	−0.90	0.14	0.15	–	–	–	–	–
GGT (U/L)	−0.39	−0.13	−0.92	0.15	0.15	–	–	–	–	–
LKCs (10^3/mm^3)	2.79	0.13	−0.43	6.01	0.09	−0.23	−0.01	−3.44	2.97	0.88
Platelets (10^3/mm^3)	0.24	0.39	0.15	0.32	<0.001	0.24	0.39	0.15	0.34	<0.001

BMI, Body Mass Index; LKC, leukocyte; AST, Aspartate Transaminase; ALT, Alanine Transaminase; GGT, Gamma-Glutamyl Transferase; HDL, High-density lipoproteins; LDL, Low-density lipoproteins.

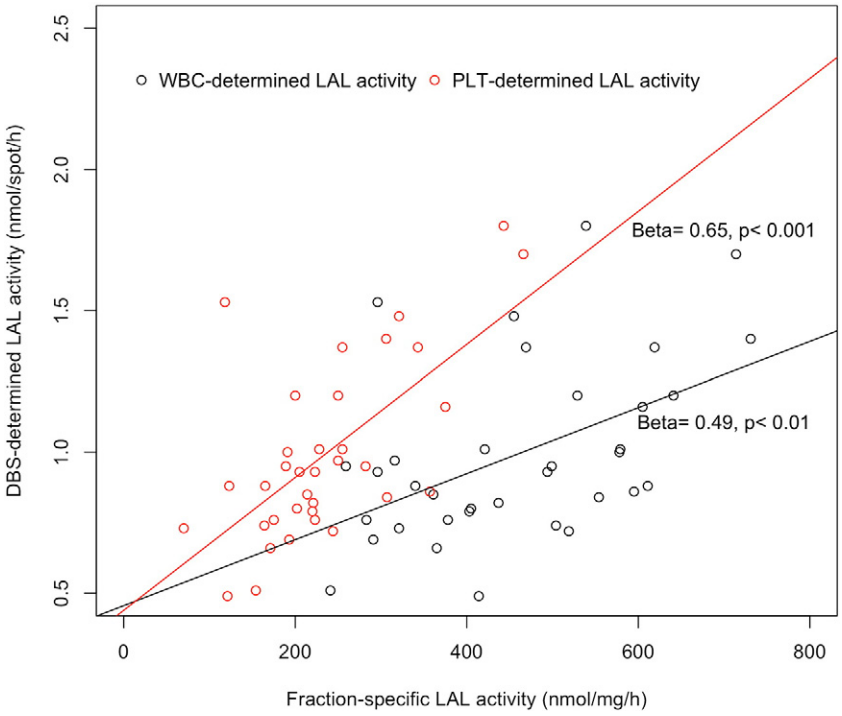

Fig. 1. Correlations between DBS-determined LAL activity and that obtained in WBCs and in PLTs. The correlation of DBS-determined LAL activity is stronger with LAL activity determined in isolated PLTs than with that determined in isolated WBCs.

cirrhotic patients but not in healthy controls, and, more interestingly, that the association with PLT count was even stronger and observed also in the control population [5]. The latter findings are robustly confirmed in the present study.

Here we decided to go further in our analysis and to evaluate LAL activity in WBCs and in PLTs. As expected, LAL function in the isolated fraction of WBCs was significantly higher than that in the PLT fraction. Based on these findings, one should expect the strongest association of LAL activity in DBS to be with the activity measured in WBCs; conversely, here we found that LAL activity measured in the PLT fraction correlated with LAL function in DBS more strongly than that determined in WBCs. Although unexpected, this result is consistent with the strong and independent association between PLT count and LAL activity in DBS, and suggests that oscillations of PLT count may impact on the result of the LAL test in DBS more than those of WBC count. More difficult is trying to understand the possible causes subtending this finding, and further investigation is certainly needed in order to clarify this point.

In conclusion, the present is the first study to demonstrate that LAL activity in DBS is strongly associated with that obtained in the PLT fraction of the blood, and that PLT number can impact on the result of the LAL DBS test. While further studies are awaited to evaluate precisely the biological determinants of these findings and, mainly, to verify if PLT count can impact on/affect other enzymatic activities determined in DBS, the present results strongly suggest consideration of PLT count when screening for LAL activity in DBS.

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