PARAOXONASE-1 L/M 55 GENE POLYMORPHISM IN THE CASES OF PRIMER IMMUNE THROMBOCYTOPENIA

ABSTRACT

Oxidative stress and free-radicals could be responsible the pathogenesis and prognosis of primer immune thrombocytopenia (ITP). Paraoxonase-1 (PON-1) is an antioxidative feature. In this study, it had investigated that the role of PON-1 Leu (L)/Met (M) 55 gene polymorphism in etiopathogenesis of ITP, course of disorder and its effects of the therapy. Here, for the first time, we described polymorphisms in patients with ITP.

51 patients with newly diagnosed ITP (ND-ITP), 15 patients with chronic ITP (CH-ITP) and 60 healthy controls (HC) were investigated.

Most common genotype in all patients with ITP, ND-ITP and CH-ITP group, HC group was LM genotype, while MM genotype was found lower in all groups. The frequency of L allel in CH-ITP group were higher. For ND-ITP group, the frequency of M allel were higher than the frequency of CH-ITP group. Individuals with a LM genotype were found to be more resistive to anti-D therapy. It is recommended to use HDMP or IVIG for patients with LL and LM genotype and only IVIG for patients with MM genotype.

PON-1 L/M 55 gene polymorphism in the cases of ITP displayed differences when compared those of controls. It could effect course of disorder, even might change response of treatment.

Key words. Primer immune thrombocytopenia, paraoxonase-1, L/M 55 gene, polymorphism

Introduction

Primer immune thrombocytopenia (ITP) is characterized by increased destruction of circulating platelets by anti-platelet antibodies. There have been many advances in our understanding of the disease pathophysiology, and some reports have implied that oxidative damage may play an important role (1). Oxidative damage may be involved in ITP pathogenesis. ITP-associated platelet destruction and bleeding may play a significant role in the elevation of lipid peroxidation and reduction in the antioxidant capacity of these patients. Paraoxonase-1 (PON-1) plays a protective role in diseases associated with oxidative stress (2). Under oxidative stress, lipoproteins, and lipids in the cellular structure undergo peroxidation. PON-1 which is synthetized in, and released from the liver is incorporated in the structure of high-density lipoprotein (HDL). It protects HDL, and low-density lipoprotein (LDL) from oxidation induced by free radicals (3, 4). PON-1 is an antioxidant enzyme which protects against deleterious effects of oxidation triggered by free oxygen radicals (5). It might be a new oxidative stress marker (6).

Two genetic polymorphisms of human serum PON-1 enzyme are detected. These two polymorphisms are formed by interchange of amino acids at 55., and 192. positions. In the first polymorphism, methionine (M allele) at 55. position is replaced by leucine (L allele) (L→M), and in the 2. polymorphism arginine (R allele) takes the place of glutamine (Q allele) (Q→R) at 192. position (7). These polymorphisms have been associated with PON-1 activity (8). L/M polymorphism effects hepatic enzyme expression, and thus its serum concentrations (9). L55 is expressed in higher amounts when compared with its M55 allele, and also L55 carriers have higher serum concentrations of PON-1. M55 is relatively less stable when compared with L55 isoform (10).

PON-1 activities may be associated with pathophysiological events of different diseases (8). In this study, it had investigated that the role of PON-1 Leu (L)/Met (M) 55 gene

polymorphism in etiopathogenesis of ITP, course of disorder and its effects of the therapy. Here, for the first time, we described polymorphisms in patients with ITP.

Materials and Methods

The study was done prospectively. 66 patients including 51 ND-ITP and 15 CH-ITP and 60 HC were enrolled in the study (Table I, II). The diagnosis of ND-ITP was made by detecting isolated thrombocytopenia (platelet counts less than 150.000/mm^3), examining bone marrow aspiration biopsy specimens, and excluding other causes of thrombocytopenia. Patients with familial thrombocytopenia, active inflammation, antinuclear antibodies, direct Coombs test positivity, and splenomegaly, and those receiving blood transfusion and/or drug therapy were excluded from the study. The term newly diagnosed ITP (ND-ITP) was defined for all cases at diagnosis (within 3 months from diagnosis). The term chronic ITP (CH-ITP) was defined for patients with ITP lasting for more than 12 months (11). Treatment to be used was selected randomly among higher doses of methylprednisolone (HDMP, 30 mg/kg/d for 3, and then 20 mg/kg/d for 4 days), standard dose prednisolone (SDP, 2 mg/kg/day, over several weeks with a taper), intravenous immune globulin (IVIG, 0.5 mg/kg for 5 days), and anti-D (50 μg /kg) (12). Accepted criteria for treatment responses were based on platelet counts as follows: 1) complete response, $\geq 100x10^9$/L; 2) partial response, 50-$100x10^9$/L; 3) mild response, $<50x10^9$/L without any need for treatment, 4) unresponsive or refractory to treatment, $<50x10^9$/L, and requirement for treatment (13). Approval from the Ethics Committee (# 1600), and informed consent of the parents were obtained.

Venous blood samples obtained after 8–12 h fasting from cases diagnosed as ITP, at the time of diagnosis were collected into tubes with K-EDTA for complete blood count, and each sample were kept frozen at -20 ^oC till the time of isolation, and analysis of their DNAs. Blood samples obtained from the control group who consulted to the healthy children polyclinic. Control group consisted of subjects without any history of ITP or other diseases,

and bone marrow depressant use. Blood samples obtained from them for any reason were subjected to the same above-mentioned conditions.

DNA purification was realized using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The primer used (oligonucleotide) in the analysis was purchased from Bio Basic firm (Bio Basic Inc., Ontario, Canada). General directions of usage were followed for the isolation of DNA. Nucleotide sequence of the purchased primer (primer sequences of PON-1 gene) manifested **F-5'**-GAA GAG TGA TGT ATA GCC CCA G-3', **R-5'**-TTT AAT CCA GAG CTA ATG AAA GCC-3 polymorphisms. Polymorphisms were determined by Restriction Fragment Length Polymorphism-Polymerase Chain Reaction (RFLP-PCR).

The fragment Hsp192 II belonging to the locus PON-1 55 was amplified by PCR, cleaved by restriction endonuclease, subjected to 2% agarose gel electrophoresis, and genotyped. PON-1 L/M 55 gene polymorphisms are shown in Figure 1 (14). As seen in Figure 2, L allele was detected at 170 bc, and M allele at 126 bc, and 44 bc bands, respectively.

Statistical analysis was performed using SPSS.12 package program. For intra-, and intergroup analyses, one way variance analysis (ANOVA), and for post-ANOVA tests LSD, and Tukey B tests were used. Differences in genotype distribution was evaluated using *chi*-square test. The significance of G, and T allele frequencies was evaluated using Fisher's Exact Test. $p<0.05$ was considered as statistically significant.

Results

Demographic characteristics of patients with ITP, and the control group are shown in Table 1. In all patients with ITP and in the groups with ND-ITP, CH-ITP and HC group, the frequency of LL genotype were founded as 24.2%, 19.6%, 40% and 26.7%, respectively. The percentages for LM genotype were 57.6%, 60.8%, 46.7%, 65% and of MM genotype were

18.2%, 19.6%, 13.3% and 8.3%. Most common genotype in all patients with ITP, ND-ITP and CH-ITP group, HC group was LM genotype, while MM genotype was found lower in all groups. The frequency of L allel in CH-ITP group were higher ($p<0.05$). For ND-ITP group, the frequency of M allel were higher than the frequency of CH-ITP group (Table 2, Figure 3).

In ND-ITP group, mean age of the patients with MM genotype was higher: than those with LL or LM genotypes, and a significant difference was found between MM, and LM genotype carriers ($p<0.05$). In CH-ITP, mean age, and platelet counts of MM carriers were lower than those detected in individuals with LL or LM genotypes ($p<0.05$). In the ND-ITP group, platelet counts were at its lowest in patients with LM, and at its highest in MM genotype carriers. However in the CH-ITP group the highest, and the lowest number of platelet counts were encountered in patients with MM, and LL genotypes, respectively (Table 3).

In patients with ND-ITP, the rates of complete, partial response, and unresponsiveness were found to be 62.5, 2.1, and 35.4%, respectively. Complete responders had LL (23.3%), LM (56.7%), and MM (20%) genotypes.

Some (22.7%) of our patients entered into chronic phase of the disease. In our patients diagnosed as CH-ITP, transient complete response (20%), partial response (33.3%), and unreponsiveness (46.7%) were observed. All the transient complete responders had LL genotypes.

Individuals with LM genotypes were more refractory to anti-D therapy. It is recommended to use HDMP or IVIG for patients with LL and LM genotype and only IVIG for patients with MM genotype (Table 4, Table 5).

Discussion

In the pathogenesis of ITP, frequently an inappropriate immune response developed postinfection has been blamed. Antibodies formed against platelet membrane or immune

conplexes induced by disease states adhere to the surface of the platelets, and lead to the destruction of young platelets. Etiologic factors triggering this autoimmunity are not clear. Among etiologic factors, cellular damage caused by hydrogen peroxide which is an oxidant product formed in association with the presence of an antibody can be enumerated (15). The role of oxidative damage in the pathogenesis of ITP has been established. Increase in lipid peroxidation, in line with a decrease in antioxidant capacity might play an important role. In ITP, levels of lipid peroxide was found to be increased, while of glutathion, and, and ascorbic acid were decreased. Higher levels of total peroxide, and comparatively lower total antioxidant capacity (TAOC) were detected. With treatment thrombocytopenia improved, and decrease in total peroxide, and increase in TAOC levels were observed. Overexpression of VNN1, an oxidative stress sensor in epithelial cells, is most strongly associated with progression to CH-ITP. Excessive production of reactive oxygen species and inadequate antioxidant capacity results in an imbalance in the redox system (16), and ITP patients are reported to have markedly higher oxidative stress levels compared to healthy individuals (17). PON activity is decreased in systemic or vascular inflammation. HDL loses the PON activity during an acute-phase response. An inverse relationship exists between serum PON activity and cardiovascular diseases. PON activity decreases in cases of chronic renal failure, inflammatory arthritis, and hypercholesterolemia (2). The TAOC level, and PON and arylesterase (ARE) enzymes activities were found lower and the total oxidant status (TOS) and oxidative stress index (OSI) levels were higher in children with ND-ITP than those in healthy children in the control group. It was also observed statistically significant increases in the TAOC, PON and ARE levels and decreases in TOS and OSI levels with 7 days of HDMP treatment compared to their values before treatment (2). The distinct patterns of gene expression in ND-ITP and CH-ITP demonstrates oxidative stress pathways in the pathogenesis of pediatric CH-ITP (15). TNF-alpha/AG, TGF-beta 1/TT, IFN-gamma/TT,

MBL/BB, and IL-1RA A1/A2 genotypes have detected as the genes of susceptibility to ITP, while TNF-alpha/AG, IFN-gamma/AA, and MBL/AB genotypes might be important in response to steroid treatment (18). PON and ARE activity levels were closely associated with the changes in the oxidative balance in pediatric ITP patients (2). Therefore, the role of PON-1 Leu (L) / Met (M) 55 gene polymorphism in ITP etiopathogenesis should be determined.

Purified, and HDL-bound PON-1 inhibits LDL oxidation. PON-1 enzyme is an active antioxidant fighting against LDL oxidation. If cellular antioxidants fail to inactivate free radicals, then these radicals interact with protein, lipid, and nucleic acids, and might induce altearions in the structure, and the functions of proteins, losses in cell membrane integration, and functions, and some mutations (19). PON-1 neutralizes the effects of lipid peroxides, and accordingly exerts a protective effect on cell membranes (20).

Paraoxonases are a multigene family of enzymes composed of three members: PON-1, PON-2, and PON-3, located adjacent to human chromosome 7 (7q21–23). In liver, and plasma PON-1, in the tissues of liver, kidney, heart, brain, testis, and aortic smooth muscle cells PON-2, and in liver, and plasma PON-3 protein are found (21). PON-1 and PON-3 are bound to HDL. PON-2 is an intracellular enzyme located in the mitochondria and endoplasmic reticulum. PON-1 binds to HDL and, in a lesser extent, can associate with VLDL and postprandial chylomicrons. PON-1 can be transported to tissues in order to exert its antioxidant function. Therefore, HDL has an anti-inflammatory effect and antioxidant properties that may prevent the oxidation of LDL (8).

PON reduces oxidative stress in serum and tissues. Because of polymorphisms, serum PON-1 activity varies 10-40-fold among individuals. In addition to this interindividual variation, because of the presence of point mutations like Q192R, and L55M, interracial differences exist as for serum PON-1 activity. In newborns the degree of serum PON activities is half of those found in adults. At postnatal 1. years normal levels are attained (22).

There is great variability in PON-1 polymorphism frequencies between individuals and populations in organophosphate metabolism. The 192R variant occurred more commonly, with a frequency of 25–64% in the populations analysed, while the 55 M allele was found to be rarer, occurring in 5–40% of the individuals. PON1 activity seems to be strongly in fluenced by the 192 Q/R polymorphisms. RR genotype carriers were evaluated “fast”, QR “intermediate” and QQ as slow metabolizers. R carriers have the highest serum paraoxonase activity. The 55 M/L polymorphism considerably affects the enzymatic activity. The 192Q/R polymorphism, MM homozygotes had lower activity towards paraoxon compared to the LM and LL genotypes (23).

It was detected that generally in patients with anticardiolipin antibody positivity, autoantibodies against LDL increase in number, while PON-1 activity decreases. Besides, R genotype which predisposes patients to a higher risk for the development of arterial thrombosis tends to increase. In IgA nephropathy lower PON-1 activity had been associated with worsening of renal functions (24, 25). PON-1 arylesterase activity was decreased in patients with sickle cell disease. No differences were observed with PON-1 L55M, and PON-2 and PON-3 polymorphisms. RR PON-1 Q192R polymorphism is likely to be a protective factor against oxidative damage in patients with sickle cell disease (8) Many studies have evaluated the association of PON-1 gene polymorphisms with enzyme activity and concentration in type 2 diabetes mellitus (T2DM). Total PON-1 activity in L55M and Q192R polymorphisms was observed differently. PON-1 enzyme activities were found higher in non-diabetic individuals in comparison to T2DM patients across different variants of L55M polymorphism but this difference was significant only in the case of LL genotype. The levels of PON-1 activity due to all of Q192R polymorphism genotypes were found significantly higher in non-diabetic subjects compared with T2DM patients (26). In T2DM, PON-1 192 BB, and PON-1 55 LL alleles were found to provide protection from oxidative

stress (27). In our study higher levels of LL genotype were detected in CH-ITP which demonstrated more intensive oxidative stress ($p<0.05$).

In our study any PON-1 activity was not detected. Only polymorphisms were studied. The presence of LM genotype supports the possibility of ND-ITP ($p<0.05$), The presence of LM genotype strongly predicts potential recovery of the patient. However MM genotype supports possible development of ITP in the affected individual ($p<0.05$).

The incidences of L and M allele in ITP, and the HC groups differed ($p<0.05$). In the ITP group L allele was less frequently detected, while M allele was encountered more often ($p<0.05$). Higher incidences of L, and M allele were noted in the CH-ITP, and ND-ITP respectively.

Individuals having LL, and LM genotypes seem to respond well to HDMP, and IVIG. Those with LM genotypes can be said to be resistant to anti-D, however MM genotype carriers are presumably more responsive to IVIG (Table 4).

Cases with CH-ITP who possessed LM genotypes did not respond to steroids, and IVIG, but responded well to vincristine. Among splenectomized cases, 2 patients had LM, and one case had LL genotype. It can be said that, possession of L allele predisposes to chronic state, but provides better treatment responses, on the contrary having M allele indicates refractoriness to treatment.

Individuals possesing MM genotype are more susceptible to infections. However those with LL genotype have a predisposition for chronic disease states, while subjects with LM genotype are more resistant to anti-D therapy. It is recommended to use HDMP or IVIG for patients with LL and LM genotype and only IVIG for patients with MM genotype.

PON-1 L/M 55 gene polymorphism in the cases of ITP displayed differences when compared those of controls. This differency might create a susceptibility in some individuals against disorder. It could effect course of disorder, even might change response of treatment.

In cases with ITP, evaluation of PON-1 Q/R 192, and L/M 55 polymorphism in conjunction with PON-1 activity will yield more illuminating results.

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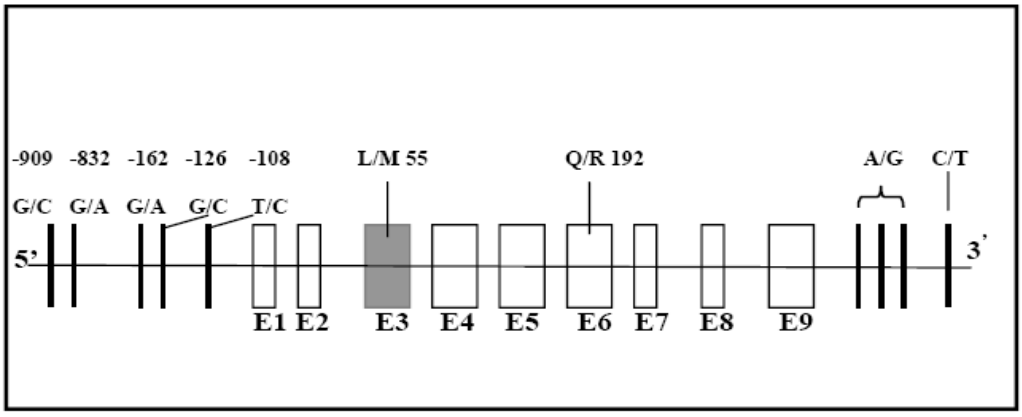

Figure 1. PON-1 L/M 55 gene polymorphisms (14)
The structure of PON-1 gene is shown with 9 exon (E1-E9) boxes. Five polymorphisms at 5' regulatory end, 2 polymorphisms in the coding region., and 4 polymorphisms at 3' untranslated end are seen.

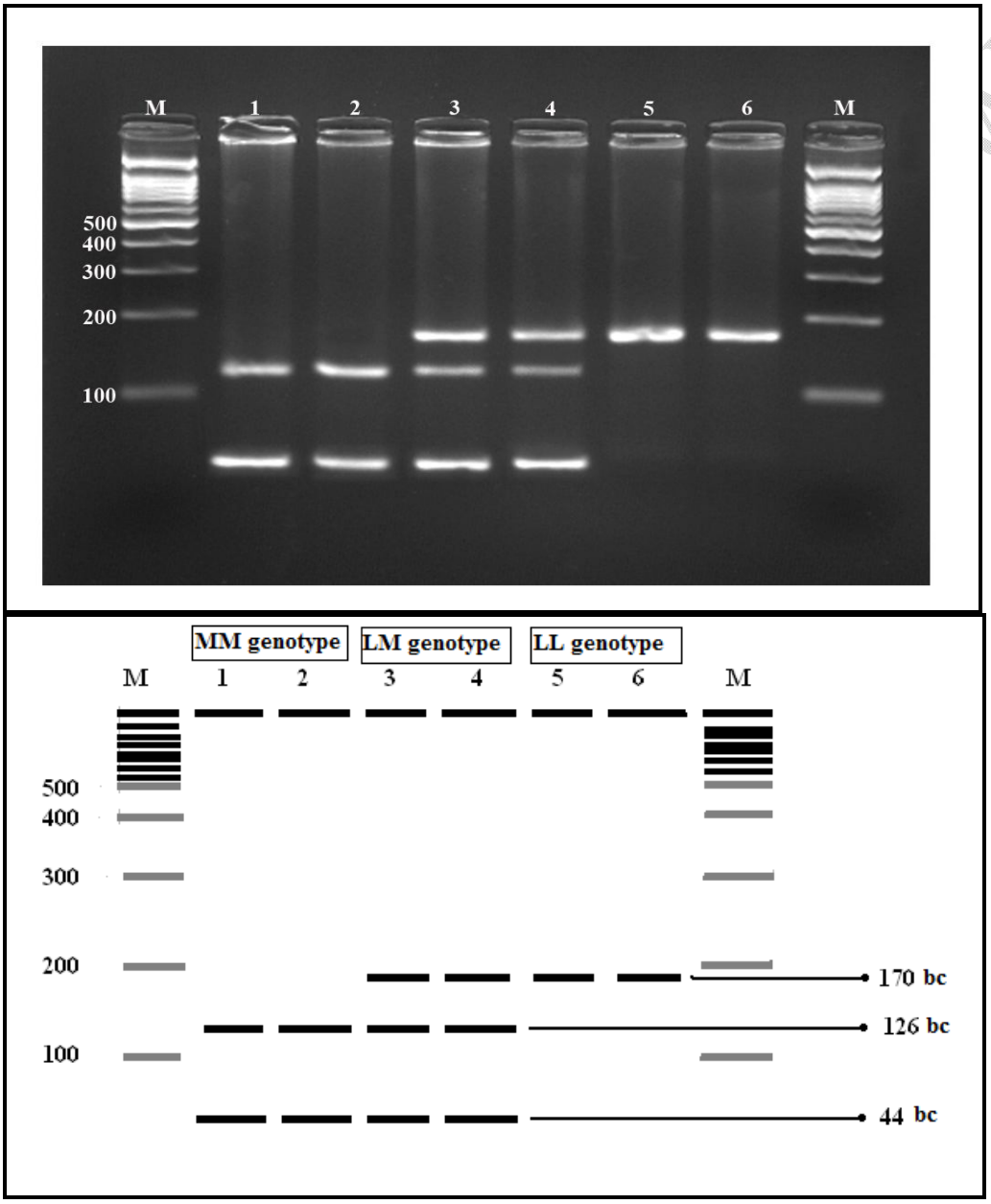

Figure 2a, 2b: PCR products cut by Hsp92 II enzyme related to PON-1 L/M 55 polymorphisms in our cases as seen in agarose gel electrophoresis.

Case 1 and 2: MM genotype, Case 3 and 4: LM genotype, Case 5 and 6: LL genotype. L allel 170 base couple (bc), M allel 126 and 44 bc. M: 100 bc DNA dimension marker

Table 1. Demographic characteristics in ITP and control groups

	Total ITP (a)	**ND-ITP (b)**	**CH-ITP (c)**	**HC (d)**	**p<0.05**
n	66	51	15	60	
Age (mean±SD,year) (min-max)	7.24±3.90 (1.5-16)	6.33±3.62 (1.5-14)	10.33±3.26 (4-16)	7.45±3.76 (1.5-16)	b-c, c-d
Gender (%)	34M/32 F (52/48)	29 M/22 F (57/43)	5 M/10 F (33/67)	39 M/21 F (65/35)	b-c, c-d

n: Patient number, **mean:** Aritmetik mean, **SD:** Standart deviation, **M:** Male, **F:** Female

Table 2. Dispersion of genotype of PON-1 L/M 55 gene polymorphism and frequency of L/M allel in the patient and control groups

	Total ITP n=66 (1)	**ND-ITP n=51 (2)**	**CH-ITP n=15 (3)**	**HC n=60 (4)**	**p<0.05**
L/M 55 dispersion of genotype					
LL (n, %)	16 (24.2)	10 (19.6)	6 (40)	16 (26.7)	**2-3, 2-4, 3-4**
LM (n, %)	38 (57.6)	31 (60.8)	7 (46.7)	39 (65)	**2-3, 3-4**
MM (n, %)	12 (18.2)	10 (19.6)	2 (13.3)	5 (8.3)	**1-4, 2-3, 2-4, 3-4**
Frequency of allel					
L (n, %)	70 (53)	51 (50)	19 (63.3)	71 (59.2)	**1-4, 2-3, 2-4**
M (n, %)	62 (47)	51 (50)	11 (36.7)	49 (40.8)	**1-4, 2-3**

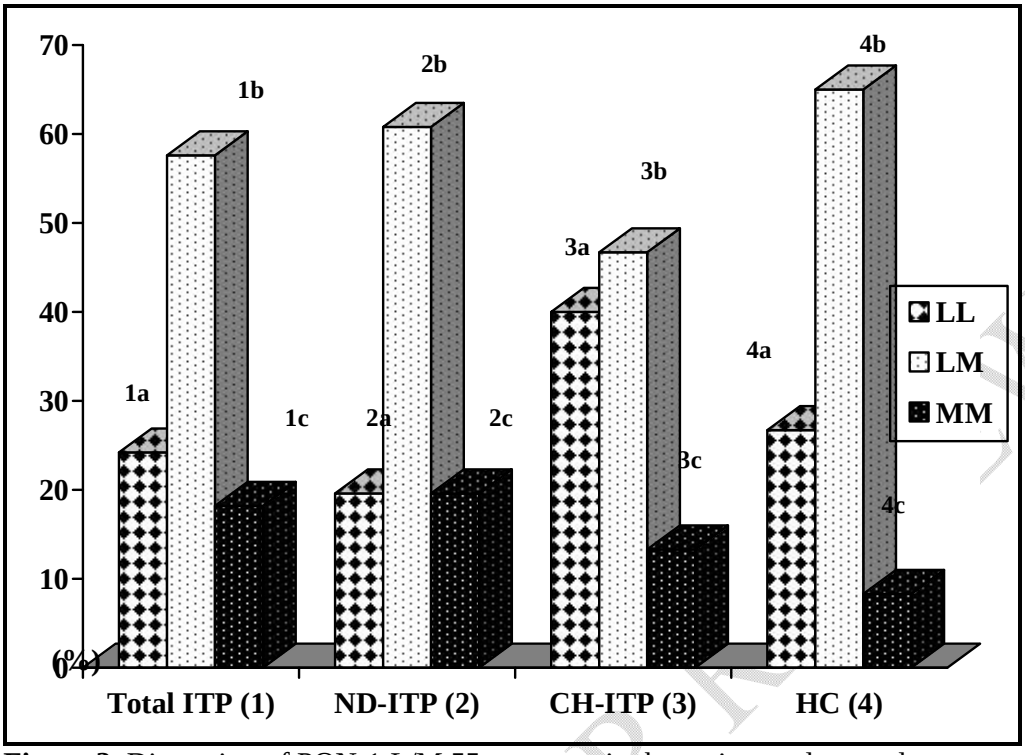

Figure 3. Dispersion of PON-1 L/M 55 genotype in the patient and control group

2a-3a= p<0.05 2b-3b= p<0.05 1c-4c= p<0.05
2a-4a= p<0.05 3b-4b= p<0.05 2c-3c= p<0.05
3a-4a= p<0.05 2c-4c= p<0.05
3c-4c= p<0.05

Table 3. Distribution of PON-1 L/M 55 genotypes among groups, and its relation with age, and thrombocytic parameters.

	ND-ITP (1)			**CH-ITP (2)**			**HC (3)**		
	LL	**LM**	**MM**	**LL**	**LM**	**MM**	**LL**	**LM**	**MM**
	n=10 (a)	**n=31 (b)**	**n=10 (c)**	**n=6 (a)**	**n=7 (b)**	**n=2 (c)**	**n=16 (a)**	**n=39 (b)**	**n=5 (c)**
Age (year) (mean±SD) (min-max)	6.25±3.77 (1.5-12)	5.53±3.13 (1.5-14)	8.9±4.04 (2.5-14)	10.91±3.72 (5.5-16)	10.42±2.33 (7.5-14)	8.25±6.01 (4-12.5)	7.53±4.15 (2-16)	7.12±3.51 (1.5-14)	9.7±4.38 (2-13)
p	1b-1c= p<0.05			2a-2c= p<0.05, 2b-2c= p<0.05			3a-3c= p<0.05, 3b-3c= p<0.05		
Platelet (10^3/mm^3) (mean±SD) (min-max)	15.10±9.93 (2.0-36.0)	12.86±13.58 (2.0-56.0)	17.50±18.75 (2.0-55.0)	30.33±33.7 (6.0-89.0)	12.14±12.5 (3.0-39.0)	5.50±0.70 (5.0-6.0)	352.50±103.70 (211.0-586.0)	326.00±71.02 (201.0-468.0)	279.80±40.27 (231.0-331.0)
p	1b-1c= p<0.05			2a-2b= p<0.05, 2a-2c= p<0.05, 2b-2c= p<0.05			AD		
MPV (fL) (mean±SD) (min-max)	11.05±2.44 (8-15.3)	11.57±3.86 (4.7-20.3)	9.69±3.44 (3.8-16.4)	12.51±1.50 (10.4-14.5)	12.28±1.32 (10.4-14.6)	15.75±6.29 (11.3-20.2)	10.61±1.92 (8.3-13)	10.52±1.53 (8.4-13.0)	10.10±1.90 (9.1-13.5)
p	AD			2a-2c= p<0.05, 2b-2c= p<0.05			AD		
PDW (mean±SD) (min-max)	34.45±21.91 (14.5-73.4)	33.69±18.24 (12.4-78.0)	33.53±17.83 (11.2-67.3)	34.23±8.92 (22.0-42.0)	33.78±6.32 (26.8-45.0)	46.0±4.24 (43.0-49.0)	28.66±6.33 (19.4-38.7)	31.0±6.60 (18.5-44.0)	35.56±5.49 (26.3-40.5)
p	AD			2a-2c= p<0.05, 2b-2c= p<0.05			3a-3c= p<0.05		

AD: p>0.05

Table 4. Treatment responses categorized according to genotypes of the patients in the ND-ITP group

ND-ITP (n= 51): LL genotype (n=10, 19.6%), LM genotype (n=31, 60.78%), MM genotype (n=10, 19.6%)
LL genotype (n=10, 19.6%): 2 (HDMP, 2 [100%] complete response), 1 (SDP, 1 [100%] unresponsive), 3 (IVIG, [100%] complete response), 4 (anti-D, 2 [50%] complete response, 1 partial response, 1 unresponsive)
LM genotype (n=31, 60.78%): 7 (HDMP, 5 [71%] complete response), 5 (SDP, 2 [40%] complete response), 10 (IVIG, 8 [80%] complete response), 9 (anti-D, [28%] complete response)
MM genotype (n=10, 19.6%): 2 (HDMP, 1 [50%] complete response), 1 (SDP, 1 [100%] complete response), 3 (IVIG, 3 [100%] complete response), 4 (anti-D, 1 [%33.3] complete response)

Table 5. Treatment responses categorized according to genotypes of the patients in the chronic ITP group

CH-ITP (n= 15): LL genotype (n=6; 40%), LM genotype (n=77 ; 46.7%), MM genotype (n=2 ; 13.3%)
Complete response (n= 3;, 20%): 3 (LL, IVIG)
Partial response (n=5, 33.3%): 3 (LL, 2 HDMP-splenectomy, 1 IVIG), 2 (LM, 2 SDP)
Refractory to treatment (n=7, 46.7%): 5 (LM, 1 HDMP-unresponsive-IVIG-unresponsive-vincristine-complete response, 1 anti-D-unresponsive-vincristine-complete response, 3 SDP-unresponsive-HDMP-unresponsive IVIG-unresponsive-vincristin-unresponsive-splenectomy), 2 (MM, 1 IVIG, 1 HDMP)