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Original Article

# The Impact of Human Platelet Antigen Allele on Antiplatelet Antibodies and Cryoglobulins in Patients with Primary Immune Thrombocytopenia and Hepatitis C Virus-Associated Immune Thrombocytopenia

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**Abstract.** ***Background And Objectives*: Human platelet antigens (HPAs) are alloantigens associated with antiplatelet alloantibodies and the risk of immune thrombocytopenia (ITP). However, few studies have investigated associations among HPAs, antiplatelet autoantibodies, and cryoglobulins.**
***Methods*: We enrolled 43 patients with primary ITP, 47 with hepatitis C virus-associated ITP (HCV-ITP), 21 with hepatitis B virus-associated ITP (HBV-ITP), 25 controls with HCV, and 1013 normal controls. We analyzed HPA allele frequencies, including HPA1-6 and 15, antiplatelet antibodies binding to platelet glycoprotein (GP) IIb/IIIa, Ia/IIa, Ib/IX, IV, human leukocyte antigen class I, cryoglobulin IgG/A/M, and their associations with thrombocytopenia.**
***Results*: In the ITP cohort, HPA2ab, rather than HPA2aa, predicted a low platelet count. HPA2b was associated with the risk of developing ITP. HPA15b was correlated with multiple antiplatelet antibodies. In HCV-ITP patients, HPA3b was correlated with anti-GPIIb/IIIa antibodies. HCV-ITP patients with anti-GPIIb/IIIa antibodies had a higher positive rate of cryoglobulin IgG and IgA compared with those without anti-GPIIb/IIIa antibodies. Overlapping detection was also found among other antiplatelet antibodies and cryoglobulins. Like the antiplatelet antibodies, cryoglobulins were associated with clinical thrombocytopenia, implying their close relationship. Finally, we extracted cryoglobulins to confirm the exhibition of cryoglobulin-like antiplatelet antibodies. In contrast, in primary ITP patients, HPA3b was correlated with cryoglobulin IgG/A/M rather than anti-GPIIb/IIIa antibodies.**
***Conclusion*: HPA alleles were associated with antiplatelet autoantibodies and had different impacts in primary ITP and HCV-ITP patients. HCV-ITP was considered to be a symptom of mixed cryoglobulinemia in HCV patients. The pathophysiology may differ between these two groups.**

**Keywords:** Antiplatelet antibody; Cryoglobulinemia; Hepatitis C virus; Human platelet antigen; Immune thrombocytopenia.

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**Introduction.** Human platelet antigens (HPAs) are alloantigens on platelet surface membrane glycoproteins (GPs). They are differentiated by single nucleotide polymorphisms in the genes encoding GPs.[1] To date, 35 antigens categorized into 29 groups have been identified,[2] among which HPA-1, HPA-2, HPA-3, HPA-4, HPA-5, and HPA-15 are biallelic, and expressed on GPIIIa, GPIba, GPIIb, GPIIIa, GPIa, and CD109, respectively. However, the frequencies of HPA alleles differ between ethnic groups and geographic areas.[3,4] These antigens are important and characterized by their immunogenicity of antiplatelet alloantibodies. HPA-associated alloantibodies have been associated with fetal and neonatal alloimmune thrombocytopenia, posttransfusion purpura, and platelet transfusion refractoriness.[1,3]

Immune thrombocytopenia (ITP) can be categorized as primary and secondary. Secondary ITP is caused by specific etiologies, like infection and autoimmune disorders. With regards to infection, hepatitis C virus (HCV) and *Helicobacter pylori* (H.p.) are well-documented causes of secondary ITP, namely hepatitis C virus-associated immune thrombocytopenia (HCV-ITP), and *Helicobacter pylori*-associated immune thrombocytopenia (H.p.-ITP). The Hepatitis B virus (HBV) has also been associated with thrombocytopenia. However, there is currently no consensus on the immune modulatory effect of HBV. Primary ITP is diagnosed by the exclusion of known etiologies.[5] The mechanism of ITP is complex and may involve the production of antiplatelet antibodies.[6] HPAs have also been associated with antiplatelet alloantibodies, and several studies have reported that certain HPAs could predict the development of ITP.[7,8] However, no reports exist on the association between HPAs and antiplatelet autoantibodies.

Cryoglobulins are serum immunoglobulins that precipitate when the temperature is cooled below 37°C, then redissolve when rewarmed. Cryoglobulinemia is classified into three types. Type I is associated with monoclonal immunoglobulin (Ig), and type II and III are mixed cryoglobulinemias associated with polyclonal IgG and monoclonal IgM or polyclonal IgM, respectively [9]. HCV is the most common etiology of mixed cryoglobulinemia, and cryoglobulinemia is a well-documented extrahepatic manifestation in chronic HCV-infected patients.[10] The clonal expansion of B cells has been observed in HCV-associated mixed cryoglobulinemia, which may be driven by antigen selection.[11] However, whether cryoglobulins bind to a specific antigen or multiple antigens is unclear. Patients with HCV-ITP have been reported to have higher rates of cryoglobulinemia and antiplatelet antibodies compared to those with primary ITP, who have higher rates than the general population.[12,13] No previous study has investigated the association between cryoglobulins and antiplatelet antibodies in ITP patients. Therefore, we conducted this study to explore the clinical associations between cryoglobulins and thrombocytopenia and evaluate the possible relationships among HPA alleles, antiplatelet antibodies, and cryoglobulins.

## Patients and Methods

*Patients*. We enrolled 111 ITP patients, 25 controls with HCV infection, and 1013 normal controls. The diagnosis of ITP was according to the American Society of Hematology guidelines when a peripheral platelet count of $< 100 \times 10^9$/L was detected.[5,14] Patients with correctable iron-deficiency anemia due to bleeding were included. A bone marrow study was performed in patients with abnormalities in peripheral blood other than thrombocytopenia and iron-deficiency anemia. Patients with thrombotic events, uncontrolled active bleeding, acute infection in the past 3 months, active cancer, and taking medications that could cause thrombocytopenia were excluded. In addition, patients with H.p.-ITP, defined as a positive urea breath test, positive endoscopic campylobacter-like organism test, or positive Giemsa staining of a stomach biopsy, were excluded. Patients positive for both serum hepatitis B surface antigen (HBsAg) and anti-hepatitis C virus antibody (anti-HCV Ab) and those with advanced cirrhosis (Child-Pugh Classification B and C) were also excluded. The enrolled patients were then classified into three groups: primary ITP (which was diagnosed by excluding known etiologies), HCV-ITP (those positive for anti-HCV Ab), and HBV-ITP (those positive for HBsAg). We also enrolled HCV control patients, who were seropositive for anti-HCV Ab and had a normal platelet count of $\geq 150 \times 10^9$/L, and a healthy volunteer group.

After enrollment, complete blood cell counts and general biochemical data, including aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, prothrombin time, bilirubin, and albumin, were collected. The platelet count was categorized into five levels: level 1, $\geq 150 \times 10^9$/L; level 2, 100 - 149 x

$10^9$/L; level 3, 50 - 99 x $10^9$/L; level 4, 30 - 49 x $10^9$/L; and level 5, < 30 x $10^9$/L. Abdominal sonography was performed to determine cirrhosis status and spleen size. Spleen size was presented as an index by multiplying the length of long and short axes over the spleen hilum at a right angle. In addition, serum and peripheral blood mononuclear cells were collected and analyzed. This study was performed in accordance with the Helsinki Declaration and approved by the Review Board at Chang Gung Memorial Hospital.

*HPA allele detection*. Peripheral blood samples from the enrolled subjects were collected in EDTA-anticoagulant tubes. Buffy coats were isolated by 700g centrifugation immediately. Peripheral blood mononuclear cells were extracted from the buffy coats by Ficoll-Hypaque gradient centrifugation (Thermo Fisher Scientific Inc., MA., USA). DNA extraction was performed by Trizol-Alcohol precipitation, and the quantity and quality of the DNA were confirmed according to a nanodrop concentration ranging from 5 to 40 ng/μl and an A260/A280 ratio between 1.65 and 2.0, respectively.

HPA typing was performed using an ExProbe SE HPA 1-6, 15, 21 Typing Kit (TBG Diagnostics Limited., Melbourne, VIC, Australia), which used sequence-specific primers with real-time polymerase chain reaction (PCR). The primer panel was mixed with pre-coated fluorescent dye on plates on an HPA typing tray according to the manufacturer's instructions. The HPA typing primers for the allele-specific sequences were amplified and detected by fluorescence activation with a specific Tm range. Simultaneously, an internal control primer was amplified with a specific Tm value. The distinct Tm values of the HPA sequence and internal control determined the HPA type and integrity of the PCR. PCR was performed using an Applied Biosystems® 7500 Real-Time PCR System according to the manufacturer's protocol. The analysis was processed using 7500 Software v2.3 with the Melting Curve function. In addition, we cooperated with the Taiwan Blood Services Foundation to include anonymous information on HPA allele frequencies in 998 Taiwanese blood donors as published by Pai et al in our healthy controls.[3]

*Cryoglobulin examination.* We used the double immunodiffusion method to identify cryoglobulin IgG, IgA, and IgM. Briefly, fresh blood samples were centrifuged at 3000 rpm for 10 min at 37°C. The collected plasma was cooled to 4ºC for 3 days and then centrifuged at 3000 rpm for 10 min at 4ºC. The deposited cryocrit at 4ºC was washed and then warmed to 37ºC for 2 hours. The dissolved cryocrit indicated the presence of cryoglobulins. Finally, the dissolved sample was mixed with anti-human IgG, IgA, and IgM and then run in agarose gel to identify the cryoglobulins. The data were presented semi-qualitatively as 1+, 2+, and 3+ by comparing with controls at a fixed concentration.

*Antiplatelet antibody detection*. We used a commercial qualitative enzyme-linked immunosorbent assay (ELISA) kit (PakPlus assay, Immucor Inc., Norcross, GA, USA) to detect antiplatelet antibodies in the collected serum. The assay detected antibodies bound to platelet surface antigens, including GPIIb/IIIa, Ia/IIa, Ib/IX, IV, and human leukocyte antigen (HLA) class I. Briefly, we added patients' serum to the wells of a 96-microwell plate coated with the platelet surface antigens aforementioned so that the present autoantibodies bound to the specific antigens. Alkaline phosphatase-labeled anti-human Ig G/A/M were then used to activate substrate p-nitrophenyl phosphate for detection. ELISA was performed according to the manufacturer's instructions.

*Examination of correlations between cryoglobulin and antiplatelet antibodies.* We selected 5 ITP patients with cryoglobulin (2+)/anti-GPIIb/IIIa antibody (+), 5 with cryoglobulin (1+)/anti-GPIIb/IIIa antibody (+). We also selected 13 controls, including 4 with cryoglobulin (2+)/anti-GPIIb/IIIa antibody (-), 5 with cryoglobulin (-)/anti-GPIIb/IIIa antibody (+), and 4 who were negative for both antibodies. To investigate whether the cryoglobulins exhibited the characteristics of antiplatelet antibodies, we extracted them using a modified version of the cryoglobulin examination method. The main difference was that we extracted the cryocrit from the frozen plasma collected for antiplatelet antibody detection instead of fresh plasm in cryoglobulin examination. The cryocrit was then manipulated as with the cryoglobulin examination method. Finally, antiplatelet antibody detection was performed for the dissolved cryocrit.

*Statistical analysis.* Differences in clinical characteristics and study variables between the ITP and control groups were evaluated using the two-sample t-test, Wilcoxon rank-sum test, or chi-square test, as appropriate. Correlations between HPA alleles and antibodies, platelet levels and antibodies, and antiplatelet antibodies and cryoglobulins were analyzed using the chi-square test and odds ratios. HPA1-5 and 15 are bi-allelic, comprised of homozygous aa/bb and heterozygous ab. Therefore, the impact of a single HPA a or b allele on antibody production was compared with a non-HPA a or b allele by weighting, with aa/bb as 2 and ab as 1. Statistical significance was defined as a two-sided *p*-value of less than 0.05. All data were analyzed using Statistical Package for the Social Sciences version 26.0 (SPSS, Chicago, IL, USA).

**Results.**

**Table 1.** Baseline characteristics of patients with different immune thrombocytopenia and the control groups

| Characteristic | Primary ITP (n =43) | HCV-ITP (n=47) | HBV-ITP (n=21) | HCV control (n=25) |
|---|---|---|---|---|
| Sex — no. (%) | | | | |
| Male | 25 (58.1) | 20 (42.6) | 10 (47.6) | 9 (36) |
| Female | 18 (41.9) | 27 (57.4) | 11 (52.4) | 16 (64) |
| Age (years) | 54.44±20.89 | 67.62*±12.97 | 60.43±12.36 | 52.52±12.43 |
| Platelet (x$10^9$/L) | 46.33*±32.56 | 48.72*±30.50 | 47.62*±31.25 | 216.36±55.06 |
| Level 1 (≥150x$10^9$/L) | N/A | N/A | N/A | 25(100) |
| Level 3 (≥50 - 99 x$10^9$/L) | 20 (46.5) | 24 (51.1) | 9 (42.9) | N/A |
| Level 4 (≥30 - 49 x$10^9$/L) | 4 (9.3) | 8 (17) | 5 (23.8) | N/A |
| Level 5 (< 30 x$10^9$/L) | 19 (44.2) | 15 (31.9) | 7 (33.3) | N/A |
| WBC (x$10^6$/L) | 6593.02±3311.27 | 4851.06*±1812.98 | 5909.52±2739.87 | 6072±1748.7 |
| Hemoglobin(g/dL) | 12.62±2.36 | 12.11*±2.18 | 12.35±1.88 | 13.43±1.30 |
| AST (U/L) | 39.05±35.61 | 51.58±35.75 | 30.67±15.70 | 40.8±25.74 |
| ALT (U/L) | 32.49*±19.63 | 48.66±41.97 | 31.33*±37.13 | 50.44±35.98 |
| Alk-p (U/L) | 70.31*±20.45 | 82.08*±31.92 | 61.14±15.23 | 52.68±10.57 |
| Albumin (g/dL) | 4.26±0.57 | 3.94*±0.56 | 4.03*±0.63 | 4.47±0.31 |
| Bilirubin (mg/dL) | 1.24±0.60 | 1.11±0.64 | 1.22±0.80 | 0.89±0.24 |
| Spleen index | 18.98*±9.15 | 22.11*±11.36 | 24.28*±13.23 | 13.70±3.62 |
| Cryoglobulinemia— no. (%) | | | | |
| Negative | 14 (45.2) | 10 (23.8) | 7 (41.2) | 11 (52.4) |
| Type I | 11 (35.5) | 9 (21.4) | 6 (35.3) | 3 (14.3) |
| Type II/III | 6 (19.4) | 23 (54.8) | 4 (23.5) | 7 (33.3) |

ITP, immune thrombocytopenia; HCV, hepatitis C virus; HBV, hepatitis B virus; WBC, white blood cell; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Alk-p, alkaline phosphatase. Data presented as a mean ± standard deviation. The differences in variants were compared with the HCV controls. *p < 0.05 denotes a significant difference.

*Baseline characteristics*. We enrolled 43 patients with primary ITP, 47 with HCV-ITP, 21 with HBV-ITP, and 25 controls with HCV infection. Their baseline characteristics are presented in **Table 1**. Compared with the HCV controls, the whole ITP cohort (primary ITP, HCV-ITP, and HBV-ITP groups combined) had a significantly larger spleen index, with the largest value (24.28) in the HBV-ITP group. The HCV-ITP patients had a higher proportion (54.8%) of type II/III mixed cryoglobulinemia, while the primary ITP patients had a lower proportion (19.4%) compared to the HCV controls (33.3%).

The results of antiplatelet antibody profiles, cryoglobulin profiles, and associated complexity in each group are presented in **Supplementary Figure 1**. Some of the antiplatelet antibody results in some patients have been reported in previous studies.[12,15] In this study, we extended the analysis to include HPA allele polymorphisms and the presence of cryoglobulins. The detection rates of total antiplatelet antibodies and cryoglobulins were higher in the HCV-ITP patients than in other groups (**Supplementary Figure 1A** and **1C**). Anti-GPIIb/IIIa antibodies and cryoglobulin IgM were these patients' most commonly detected immunoglobulins. In the complexity analysis of the three ITP groups, the HBV-ITP group had the lowest rates of the presence of three or more types of antiplatelet antibodies and cryoglobulins (**Supplementary Figure 1B and 1D**).

*HPA2ab was associated with lower platelet count, and HPA2b was associated with the risk of developing ITP.* After including the anonymous HPA polymorphism data of 998 Taiwanese blood donors in addition to our 15 normal controls, there were a total of 1013 normal controls. Regarding the heterogenicity of HPA polymorphisms, HPA3 and HPA15 were the most heterogenous in our population (**Table 2**). The HBV-ITP patients had a significantly lower proportion of HPA15ab and a higher proportion of HPA15aa (p=0.047, **Table 2**). In the analysis of specific allele frequencies, the whole ITP cohort had a higher HPA2b allele frequency than the normal controls (6.3% versus 3.5%, p=0.038, **Table 3**), and this trend was observed in all three ITP groups. Therefore, we further explored the HPA alleles and clinical presentations. The results showed that HPA2ab was associated with a higher rate of severe thrombocytopenia (level 5 platelet count of <30 x$10^9$/L, p=0.023, **Supplementary Figure 2A**). In addition, the whole ITP cohort with HPA2ab had a significantly lower mean platelet count than those with HPA2aa (31.36 x$10^9$/L versus 49.93 x$10^9$/L, p=0.037,

**Table 2.** Human platelet antigen polymorphism in different groups.

| | HPA1 | | HPA2 | | | HPA3 | | | HPA4 | | HPA5 | | HPA6 | | | HPA15 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | aa | ab | aa | ab | bb | aa | ab | bb | aa | ab | aa | ab | aa | ab | bb | aa | ab | bb |
| Primary ITP-no. | 43 | 0 | 38 | 5 | 0 | 16 | 22 | 5 | 43 | 0 | 42 | 1 | 41 | 2 | 0 | 12 | 24 | 7 |
| % | 100.0 | 0.0 | 88.4 | 11.6 | 0.0 | 37.2 | 51.2 | 11.6 | 100.0 | 0.0 | 97.7 | 2.3 | 95.3 | 4.7 | 0.0 | 27.9 | 55.8 | 16.3 |
| HCV-ITP-no. | 46 | 1 | 41 | 6 | 0 | 15 | 23 | 9 | 46 | 1 | 46 | 1 | 46 | 1 | 0 | 14 | 26 | 7 |
| % | 97.9 | 2.1 | 87.2 | 12.8 | 0.0 | 31.9 | 48.9 | 19.1 | 97.9 | 2.1 | 97.9 | 2.1 | 97.9 | 2.1 | 0.0 | 29.8 | 55.3 | 14.9 |
| HBV-ITP-no. | 21 | 0 | 18 | 3 | 0 | 8 | 9 | 4 | 21 | 0 | 21 | 0 | 20 | 1 | 0 | 11 | 6 | 4 |
| % | 100.0 | 0.0 | 85.7 | 14.3 | 0.0 | 38.1 | 42.9 | 19.0 | 100.0 | 0.0 | 100.0 | 0.0 | 95.2 | 4.8 | 0.0 | 52.4* | 28.6* | 19.0* |
| HCV control-no. | 25 | 0 | 24 | 1 | 0 | 8 | 12 | 5 | 25 | 0 | 24 | 1 | 25 | 0 | 0 | 4 | 15 | 6 |
| % | 100.0 | 0.0 | 96.0 | 4.0 | 0.0 | 32.0 | 48.0 | 20.0 | 100.0 | 0.0 | 96.0 | 4.0 | 100.0 | 0.0 | 0.0 | 16.0 | 60.0 | 24.0 |
| Normal control-no. | 1004 | 9 | 944 | 67 | 2 | 301 | 529 | 183 | 1008 | 5 | 982 | 31 | 968 | 44 | 1 | 288 | 513 | 212 |
| % | 99.1 | 0.9 | 93.2 | 6.6 | 0.2 | 29.7 | 52.2 | 18.1 | 99.5 | 0.5 | 96.9 | 3.1 | 95.6 | 4.3 | 0.1 | 28.4 | 50.6 | 20.9 |

ITP, immune thrombocytopenia; HCV, hepatitis C virus; HBV, hepatitis B virus; H.p., *helicobacter pylori*; HPA, human platelet antigen. *p < 0.05 denotes significant difference between HBV-ITP and normal control by Pearson chi-square test.

**Table 3.** Human platelet antigen alleles frequency in different groups.

| | HPA1 | | HPA2 | | HPA3 | | HPA4 | | HPA5 | | HPA6 | | HPA15 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | a | b | a | b | a | b | a | b | a | b | a | b | a | b |
| Total ITP | 99.5 | 0.5 | 93.7 | 6.3* | 59.5 | 40.5 | 99.5 | 0.5 | 99.1 | 0.9 | 98.2 | 1.8 | 58.6 | 41.4 |
| Primary ITP | 100.0 | 0.0 | 94.2 | 5.8 | 62.8 | 37.2 | 100.0 | 0.0 | 98.8 | 1.2 | 97.7 | 2.3 | 55.8 | 44.2% |
| HCV-ITP | 98.9 | 1.1 | 93.6 | 6.4 | 56.4 | 43.6 | 98.9 | 1.1 | 98.9 | 1.1 | 98.9 | 1.1 | 57.4 | 42.6% |
| HBV-ITP | 100.0 | 0.0 | 92.9 | 7.1 | 59.5 | 40.5 | 100.0 | 0.0 | 100.0 | 0.0 | 97.6 | 2.4 | 66.7 | 33.3% |
| HCV control | 100.0 | 0.0 | 98.0 | 2.0 | 56.0 | 44.0 | 100.0 | 0.0 | 98.0 | 2.0 | 100.0 | 0.0 | 46.0 | 54.0% |
| Normal control | 99.6 | 0.4 | 96.5 | 3.5 | 55.8 | 44.2 | 99.8 | 0.2 | 98.5 | 1.5 | 97.7 | 2.3 | 53.8 | 46.2% |

ITP, immune thrombocytopenia; HCV, hepatitis C virus; HBV, hepatitis B virus; HPA, human platelet antigen. *p < 0.05 denotes significant difference between total ITP patients and normal control by Pearson chi-square test.

**Supplementary Figure 2B**). These results showed that HPA2b was associated with the risk of ITP and clinical thrombocytopenia. Because HPA2 determines the alloantigen on GPIb, we further explored the association between HPA2a/2b and anti-GPIb/IX antibodies. The results showed no significant difference in the HPA2b incidence between the patients with or without anti-GPIb/IX antibodies.

*HPA15 was associated with the production of many types of antiplatelet antibodies.* We also explored the possible relationships of the most heterogeneous HPA alleles, HPA3 and HPA15, with antiplatelet antibodies and cryoglobulins in the complete ITP cohort. The results showed that HPA15b was associated with higher positive rates of anti-GPIa/IIa, Ib/IX, IV, and HLA Class 1 antibodies compared with non-HPA15b, whereas HPA15a was inversely associated with anti-GPIb/IX, IV, and HLA Class 1 antibodies compared with non-HPA15a (**Figure 1A**). It suggested that ITP patients with HPA15b would have more complex antiplatelet antibody profiles than those without HPA15b, while ITP patients with HPA15a would have more simple profiles than those without HPA15a. There was no obvious association between HPA15 and cryoglobulins. Because HPA alleles consist of aa, ab, and bb, the comparisons between a/b versus non-a/b and between a versus b make different results. In the direct comparison between HPA15b versus HPA15a on antiplatelet antibody production, the results still showed a significantly higher risk with HPA15b than HPA15a for the production of anti-GPIb/IX, GPIV, and HLA Class I antibodies at odds ratios of 2.452, 3.841, and 1.939, respectively (Supplementary **Table 1**). These results reflected the detected number of antiplatelet antibody types in HPA15 alleles. As shown in **Figure 1B**, the detection rates of 2 and ≥ 3 types of antiplatelet antibodies were higher in HPA15b than in HPA15a. In short, HPA15b was associated with risk for multiple antiplatelet antibodies compared with HPA15a.

*HPA3 was associated with the production of anti-GPIIb/IIIa antibodies and cryoglobulins.* In the complete ITP cohort, HPA3b was positively associated with cryoglobulin IgG/IgM and total cryoglobulins production compared with non-HPA3b, whereas HPA3a

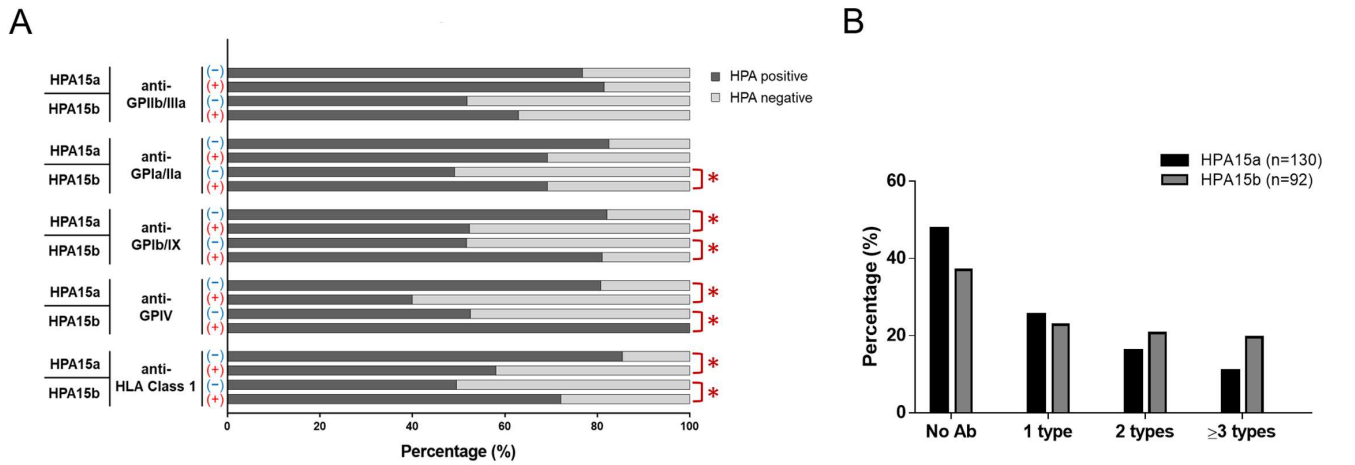


**Figure 1.** A. Associations of HPA15 alleles and detection of antiplatelet antibodies. Patients with HPA15b had significantly higher positive detection rates of anti-GPIa/IIa, Ib/IX, IV, and HLA Class 1 antibody compared to those without HPA15b (p=0.016, 0.017, 0.002, and 0.011, respectively). On the other hand, HPA15a was inversely associated with the production of anti-GPIb/IX, IV, and HLA Class 1 antibody (p=0.002, 0.006, and <0.001, respectively). The analysis was performed using Pearson's method with weighting. * denotes a significant difference. B. Antiplatelet antibody complexity between HPA15a and HPA15b. For HPA15a, the antiplatelet antibody detection rates decreased persistently as the antiplatelet antibody complexity increased. On the other hand, the detection rates were similar as complexity increased in HPA15b. As a result, the detection rates of the undetected and only 1 type of antiplatelet antibody were higher in HPA15a than in HPA15b, while those of 2 types and ≥ 3 types of antiplatelet antibodies were lower inversely in HPA15a compared with HPA15b.

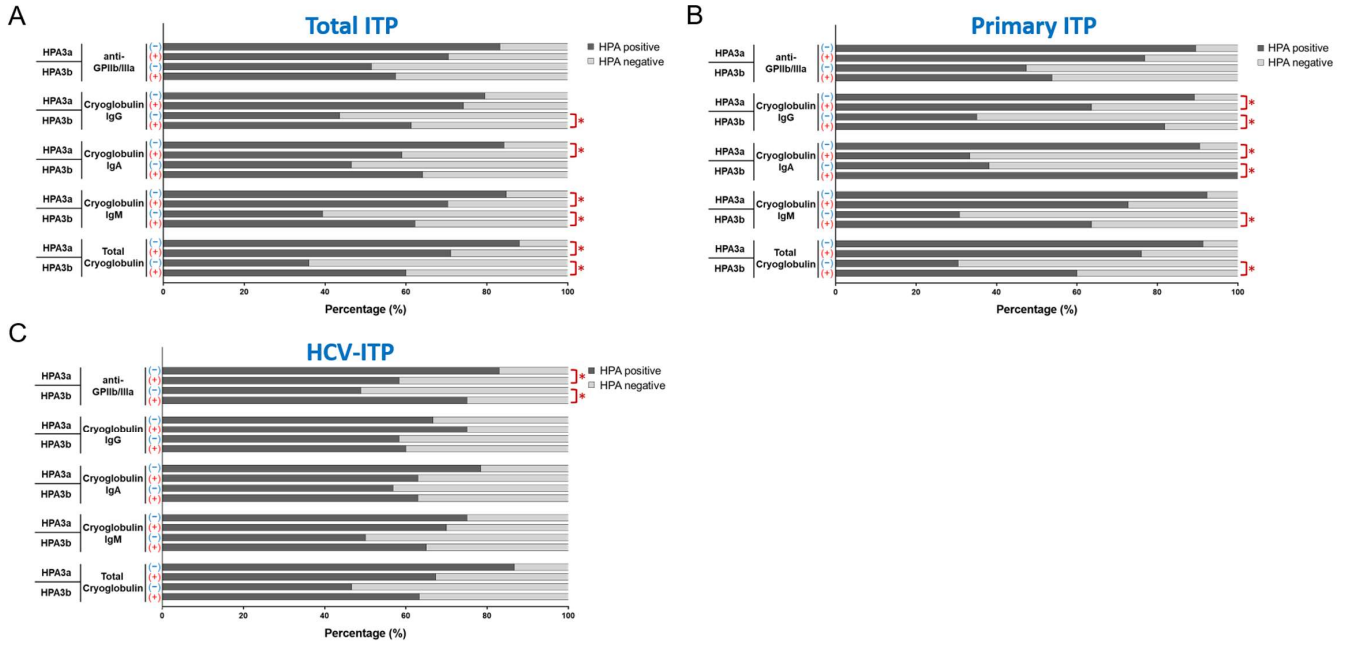


**Figure 2.** Correlations of HPA3 alleles with anti-GPIIb/IIIa antibodies and cryoglobulins. A. In the whole ITP cohort, HPA3b was positively associated with cryoglobulin IgG, IgM, and total cryoglobulins (p=0.037, 0.007, and 0.006, respectively), whereas HPA3a was inversely associated with cryoglobulin IgA, IgM, and total cryoglobulins (p=0.001, 0.004, and 0.023, respectively). B. In primary ITP patients, HPA3b was correlated with cryoglobulin IgG/A/M and total cryoglobulins (p=0.006, 0.006, 0.023, and 0.024, respectively). On the other hand, HPA3a was inversely associated with cryoglobulin IgG and IgA (p=0.046 and 0.005, respectively). C. In HCV-ITP patients, HPA3b was only positively associated with the production of anti-GPIIb/IIIa antibodies (p=0.035). In contrast, HPA3a was inversely associated with anti-GPIIb/IIIa antibodies (p=0.024). The analysis was performed using Pearson's method with weighting. * denotes a significant difference.

was inversely associated with cryoglobulin IgA/IgM and total cryoglobulins production compared with non-HPA3a (**Figure 2A**). Similarly, HPA3b had a higher risk for cryoglobulin IgA and IgM compared to HPA3a at odds ratios of 1.966 and 1.905, respectively (Supplementary **Table 2**). In contrast, there was no significant association between HPA3a/3b and anti-GPIIb/IIIa antibodies. Because HPA3 is an important alloantigen on GPIIb, the results could have been clearer. To exclude the possible different pathophysiology of each ITP group, we further analyzed the effects of HPA3a/3b on anti-GPIIb/IIIa antibodies and cryoglobulins in the primary ITP and HCV-ITP patients, respectively. In the primary ITP patients, HPA3b was associated with cryoglobulin IgG/A/M production, but HPA3a was inversely correlated (**Figure 2B**). On the

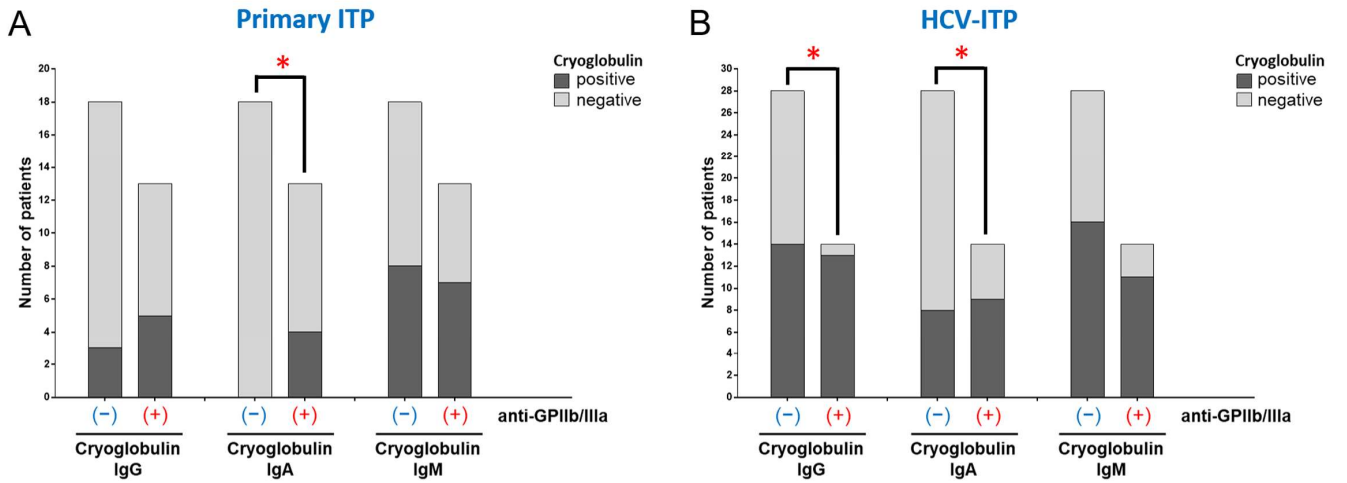


**Figure 3.** Correlations between anti-GPIIb/IIIa antibodies and cryoglobulin. A. Primary ITP patients with anti-GPIIb/IIIa antibodies had significantly higher positive detection for cryoglobulin IgA than those without anti-GPIIb/IIIa antibodies (p=0.023). All patients with cryoglobulin IgA were positive for anti-GPIIb/IIIa antibodies. B. HCV-ITP patients with anti-GPIIb/IIIa antibodies had higher positive detection for cryoglobulin IgG and IgA than those without anti-GPIIb/IIIa antibodies (p=0.007 and 0.026, respectively). Most of the patients with anti-GPIIb/IIIa were positive for cryoglobulin IgG/A/M. * denotes a significant difference. (-) and (+) indicated negative and positive for anti-GPIIb/IIIa antibody.

other hand, in the HCV-ITP patients, HPA3b was associated with anti-GPIIb/IIIa antibodies detection, whereas HPA3a was inversely associated with anti-GPIIb/IIIa antibodies (**Figure 2C**). Although the associations of HPA3b on anti-IIb/IIIa antibody in primary ITP and HPA3b on cryoglobulin IgG/A/M in HCV-ITP did not reach statistical significance, a trend of the associations still exists.

*Positive correlations between anti-GPIIb/IIIa antibodies and cryoglobulin detection.* Because HPA3 alleles were associated with anti-GPIIb/IIIa antibodies and cryoglobulins production, we hypothesized that there might be associations between these immunoglobulins. We first explored the overlapping incidence between these two categories of immunoglobulins. The primary ITP patients with anti-GPIIb/IIIa antibodies had a higher positive rate of cryoglobulin IgA than those without anti-GPIIb/IIIa antibodies (p=0.023, **Figure 3A**). On the other hand, the HCV-ITP patients with anti-GPIIb/IIIa antibodies had significantly higher rates of positive cryoglobulin IgG and IgA than those without anti-GPIIb/IIIa antibodies (p=0.006 and p=0.026, respectively, **Figure 3B**). Although anti-GPIIb/IIIa antibodies were not statistically associated with cryoglobulin IgM, most HCV-ITP patients with anti-GPIIb/IIIa antibodies similarly had a high presentation of cryoglobulin IgM (**Figure 3B**). In addition, most HCV-ITP patients with any one positive antiplatelet antibody had a positive detection for cryoglobulins. Conversely, the HCV-ITP patients without cryoglobulin nearly had no detectable antiplatelet antibody (**Supplementary Table 3**). Accordingly, the detection of both types of immunoglobulins overlapped in the HCV-ITP patients but not in the primary ITP patients. It suggested that cryoglobulins may comprise multiple antiplatelet antibodies in HCV-ITP patients. These findings also reflected that HPA3 was specifically associated with anti-GPIIb/IIIa antibodies rather than cryoglobulins in the HCV-ITP patients.

*Antiplatelet antibodies and cryoglobulins were significantly associated with platelet levels in HCV-ITP patients.* Because of the high degree of overlap between antiplatelet antibodies and cryoglobulins, we further explored the clinical impact of these two categories of antibodies. The antiplatelet antibody profiles and complexity in the HCV-ITP patients and HCV controls according to platelet level are shown in **Figures 4A** and **4B**. Antiplatelet antibodies' rates and complexity increased as the platelet count decreased. Among them, the increases in the rates of anti-GPIb/IX and total antiplatelet antibodies reached significance (p=0.009 and 0.005, respectively). Similarly, the cryoglobulin IgG, IgA, and IgM detection rates increased as the platelet count decreased, of which cryoglobulin IgA reached statistical significance (p=0.018, **Figure 4C**). For cryoglobulin complexity, the positive rates of the three types of cryoglobulins increased as the platelet count decreased, whereas the rate of undetectable cryoglobulin decreased as the platelet count decreased (**Figure 4D**). However, in the primary ITP patients, correlations between the detection rates of antiplatelet antibodies and cryoglobulins with platelet levels were not found (data not shown). Although the primary ITP patients with cryoglobulin IgA had a lower mean platelet count (33 $x10^9$/L) than those without IgA (50.11 $x10^9$/L), the number of cases was too small to make definitive

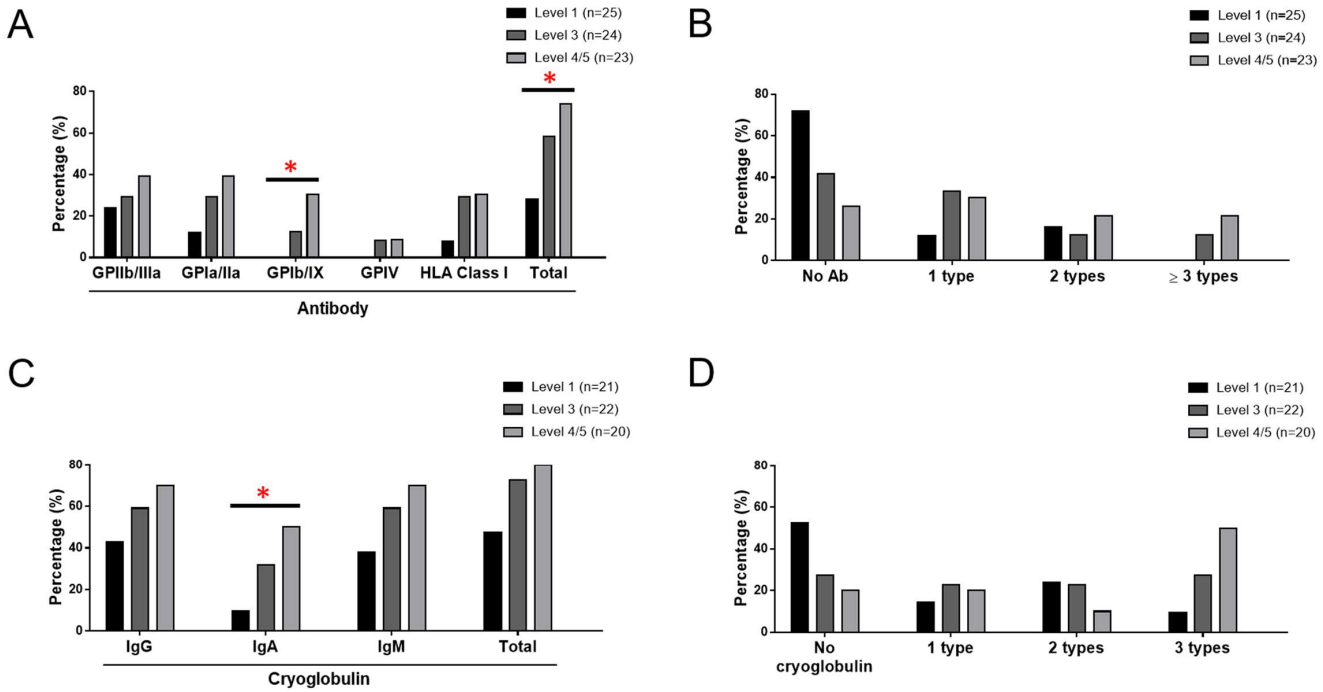


**Figure 4.** Antiplatelet antibody and cryoglobulin results according to platelet levels in HCV patients. A. Antiplatelet antibody profiles by platelet level. Antiplatelet antibodies showed a trend of increasing detection rate as the platelet count decreased, of which anti-GPIb/IX antibody and total antiplatelet antibody positive rates reached statistical significance (p=0.009 and p=0.005, respectively). B. Antiplatelet antibody complexity by platelet level. Non-detection rates decreased as platelet count decreased. In contrast, the detection rate of more than three types of antiplatelet antibodies increased as the platelet count decreased. C. Cryoglobulin profile by platelet level. Cryoglobulin detection showed a similar trend to antiplatelet antibody detection, with a higher positive rate as the platelet count decreased, of which cryoglobulin IgA reached significance (p=0.018). D. Cryoglobulin complexity by platelet level. Similar to antiplatelet antibody complexity, the non-detection rates decreased and positive rates of the three types of cryoglobulins increased as the platelet count decreased. * denotes a significant difference.

conclusions. These findings demonstrated that both antiplatelet antibodies and cryoglobulins were significantly correlated with platelet count in HCV patients.

*Cryoglobulins showed characteristics of antiplatelet antibodies.* We extracted the cryocrit from blood samples to detect the presence of antiplatelet antibodies. The ITP patients with positive anti-GPIIb/IIIa antibodies always had other antiplatelet antibodies. The data showed that the patients with cryoglobulin (2+)/anti-GPIIb/IIIa antibody (+) had significantly higher detection ratios of not only anti-GPIIb/IIIa antibodies but also anti-GPIa/IIa, Ib/IX, and IV antibodies than controls (Supplementary **Figure 3**). These results confirmed that the cryoglobulins showed characteristics of antiplatelet antibodies in the primary ITP and HCV-ITP patients who were strongly positive for cryoglobulins and antiplatelet antibodies. However, these patients were much fewer in primary ITP than HCV-ITP patients. These findings are summarized and illustrated in Supplementary **Figure 4**.

**Discussion.** This study demonstrated correlations among HPA alleles, antiplatelet antibodies, and cryoglobulins in primary and secondary ITP patients. HPA alleles have been reported to be a risk factor for the development of ITP. Of HPA1-5 and 15 alleles, HPA2 has been reported to be most strongly associated with the development of ITP.[7,8,16-18] Consistently with studies conducted in Egypt and Macedonia,[7,18] we echoed that HPA2b was a significant risk for ITP development. We further found that the ITP patients with HPA2b had lower platelet counts than those without HPA2b. The presence of anti-GPIb/IX antibodies was also significantly correlated with the level of thrombocytopenia. A few alloantigens are located on GPIb, of which HPA2 is the most important.[1] Therefore, HPA2b-associated anti-GPIb/IX antibodies might contribute to the risk for ITP. However, we found no correlation between HPA2b and anti-GPIb/IX antibody production, but the limited number of cases limits the ability to make definite conclusions. There is another possibility that HPA2b may affect the epitope specificity and the production of anti-GPIb/IX antibodies.

HPA15 alloimmunization has been documented in fetal/neonatal alloimmune thrombocytopenia and platelet transfusion refractoriness but was rarely detected.[19-21] However, this is the first study to show that HPA15b was significantly associated with multiple non-epitope antiplatelet antibody production compared to HPA15a. There were three main findings. First, HPA15 was strongly associated with anti-HLA class I and pan-antiplatelet antibodies. Second, cryoglobulins were not associated with HPA15, becoming a negative control. Third, HPA15 is located on CD109, the only platelet membrane GP mainly expressed on the surface of

platelets and either white blood cells, especially T lymphocytes.[1,22] CD109 has been reported to be a coreceptor of transforming growth factor-beta, which inhibits signal pathways and affects the immune response. Therefore, we hypothesize that HPA15b may promote the alloimmunization of multiple GP alloantigens, enhancing the complexity of antiplatelet antibody production. Significantly the HBV-ITP patients had lower HPA15b and lower heterogenicity of antiplatelet antibodies.

Anti-GPIIb/IIIa antibodies were this study's most frequently detectable antiplatelet antibodies, consistent with a previous study.[23] HPA3 was the most heterogenous HPA polymorphism, as reported in many other ethnicities.[4] HPA3 is present on the most prevalent GPIIb on the platelet cell membrane.[1] Therefore, the anti-GPIIb/IIIa antibodies may be related to the high prevalence of GPIIb/IIIa and heterogeneity of the located alloantigens. Furthermore, we found that HPA3b was positively associated with anti-GPIIb/IIIa antibodies and cryoglobulin production but had different impacts in the patients with primary ITP and HCV-ITP. Most of the HCV-ITP patients with antiplatelet antibodies were positive for cryoglobulins. Clinically, cryoglobulins, especially IgA, similar to antiplatelet antibodies, had an impact on thrombocytopenia. These results imply that a proportion of cryoglobulins may share the function of antiplatelet antibodies in HCV-ITP patients. Then we confirmed this hypothesis by directly extracting cryoglobulins from patient samples to detect the existence of antiplatelet antibodies. Accordingly, a large proportion of HPA3b-associated anti-GPIIb/IIIa antibodies are a part of total cryoglobulins. That explains why HPA3b is specifically correlated with anti-GPIIb/IIIa antibodies rather than cryoglobulins in HCV-ITP patients. On the other hand, HPA3b was specifically associated with cryoglobulins IgG/A/M in the primary ITP patients. In the primary ITP patients, only some anti-GPIIb/IIIa antibodies are HPA3-associated cryoglobulin-like antibodies, leading to the correlation between HPA3 and cryoglobulins rather than anti-GPIIb/IIIa antibodies. These results are summarized and illustrated in **Supplementary Figure 4**.

Mixed cryoglobulinemia has been reported to be an extrahepatic manifestation in HCV patients. This study presented a high detection rate of cryoglobulinemia in HCV-ITP patients, as reported in a previous landmark study.[13] In addition, a good correlation between antiplatelet antibodies and cryoglobulinemia was reported in this study. Regarding the pathophysiology, mixed cryoglobulinemia has been reported to be an antigen-derived immune modulator.[11,24] HCV may induce autoimmunization to many alloantigens and the production of autoantibodies, leading to mixed cryoglobulinemia. Taken together, we hypothesize that HPA is one of the complex alloantigens which induce cryoglobulin-like antiplatelet antibodies. These immunoglobulins are associated with clinical thrombocytopenia, causing a certain proportion of HCV-ITP to be a symptom of mixed cryoglobulinemia in HCV patients, as seen in this study. Cryoglobulin-like antiplatelet antibodies' detection rate and complexity were closely associated with platelet count. On the other hand, the pathophysiology of antiplatelet antibody production has been well-documented in primary ITP patients.[25] Primary ITP patients mainly have T-cell immune modulation and specific antiplatelet antibody formation. Although HPA-associated cryoglobulins may share the characteristics of antiplatelet antibodies to some degree, the impact of these HPA allele-derived cryoglobulin-like antiplatelet antibodies was much less prominent and important in primary ITP than in the HCV-ITP patients, as shown in this study. Actually, it also reflected the lower positive rates of antiplatelet antibodies and cryoglobulins in primary ITP patients compared with HCV-ITP patients, suggesting that different treatment strategies may be required for patients with these two kinds of ITPs. However, there may also be similarities between these two ITPs. The roles of HPA2b, cryoglobulin IgA, and HPA15b seem to be important for thrombocytopenia and antiplatelet antibody formation.

There are some limitations to this study. Although we comprehensively demonstrated strong correlations among HPA, antiplatelet autoantibodies, cryoglobulins, and associated clinical impact on antiplatelet count, the definite pathophysiology of how HPA alleles affect platelet antibodies and cryoglobulin production needed to be clarified. Second, the case number in each study group was relatively small. However, most of the results were demonstrated in the complete ITP cohort, including the effect of the HPA alleles on the risk of ITP or antiplatelet antibody/cryoglobulin production. The impact of the b allele of HPA2, HPA3, and HPA15 on antiplatelet antibodies and cryoglobulins was consistent in the whole ITP cohort and subgroups.

**Conclusions**. The antiplatelet antibodies pathophysiology and associated characteristics differed in the primary ITP and HCV-ITP patients. This may lead to different management strategies and responses to treatment. ITP may be considered a complication of cryoglobulinemia in a certain proportion of HCV patients.

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**Ethics approval.** This study was conducted according to the Helsinki Declaration and was approved by the Institutional Review Board of Chang Gung Memorial Hospital. This article does not contain any studies with animals performed by any of the authors.

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