Immune thrombocytopenia: exploring antibodies, scintigraphy and immune modulation. Moving towards a new era for patients with ITP

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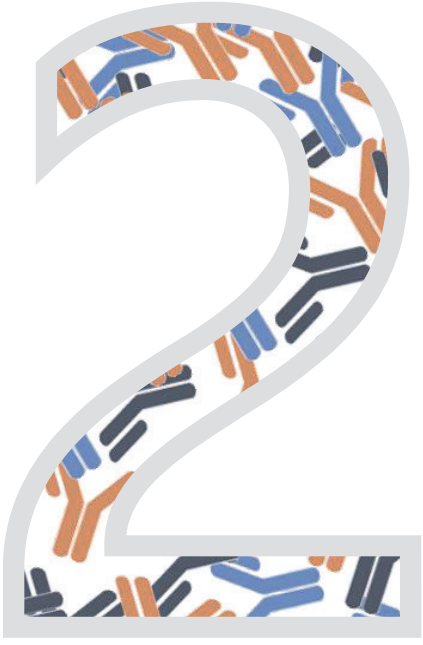

Chapter 2

Anti-Glycoprotein Antibodies and Sequestration Pattern of Indium Labeled Platelets in Immune Thrombocytopenia

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Abstract

Background Anti-glycoprotein antibodies play an important role in the pathophysiology of immune thrombocytopenia (ITP). The sequestration pattern of platelets in spleen and liver can be studied with Indium-111 labeled autologous platelet scans. No studies have investigated the role of anti-GP antibodies in sequestration pattern in ITP patients. In this study we examined the association between antibodies and 1) platelet sequestration site and 2) clearance rate of platelets.

Methods All ITP patients receiving an Indium-111 labeled autologous platelet study between 2014 and 2018 were included. Antibodies were measured using the direct MAIPA method to determine the presence and titer of anti-GPIIb/IIIa, anti-GPIb/IX and anti-GPV antibodies. Multivariate regression models were used to study the association between anti-GP antibodies, sequestration site and clearance rate.

Results Seventy-four patients were included, with a mean age of 36 years. Forty-seven percent of the patients showed a predominantly splenic sequestration pattern, 29% mixed and 25% a hepatic pattern. In 53% of the patients anti-GP antibodies were detected. Regression models showed a significant association between splenic sequestration and GPV autoantibodies.. Furthermore, in patients where antibodies were present the clearance rate was higher in patients with a splenic sequestration.

Conclusion Anti-GPV antibodies are associated with a splenic sequestration pattern in ITP patients. These associations provide insight in the possible pathophysiological mechanisms of ITP, which may lead to better detection and treatment of this partly idiopathic and prevalent disease.

Key Points

- Anti-GPV antibodies are associated with a splenic sequestration pattern in this cohort.
- In the presence of antibodies, platelet clearance rate was associated with splenic sequestration.

Introduction

Immune thrombocytopenia (ITP) is an acquired auto immune disorder characterized by low platelet numbers (peripheral blood platelet count <100 × 10^9/L). The thrombocytopenia results from increased clearance of platelets combined with impaired production of platelets. (1) ITP is generally manifested by an increased bleeding tendency ranging from minor skin manifestations with petechiae and/or purpura, to mucosal bleedings and possible fatal intracranial hemorrhages. In addition, patients with ITP experience a lower quality of life and difficulties in continuing their normal life routine, such as work. (2)

The pathophysiology of ITP is heterogeneous and partly unknown. Several pathways have been described in the development and chronicity of ITP. The most important mechanisms are 1) antibody mediated platelet and/or megakaryocyte destruction, clearance and inhibited production, and 2) T- and NK cell activity against platelets and/or megakaryocytes. (3-6) When autoantibodies are detected in ITP, the majority of those autoantibodies are directed against epitopes on glycoprotein (GP) IIb/IIIa, Ib/IX and/or V. (6-8) Megakaryocytes (MKs) are inhibited in their pro-platelet formation and platelet-release by autoantibodies. (9, 10) These autoantibodies can lead to both splenic and hepatic clearance of platelets. Antibodyopsonized platelets may be recognized by FcγR bearing phagocytes leading to mostly splenic clearing of platelets. Anti-GPIIb/IIIa is the most detected antibody in immune thrombocytopenia; leading to splenic removal of platelets. It was reported that anti-GPIb/IX antibodies may lead to accelerated desialylation of platelets and subsequently earlier recognition by the Ashwell Morell Receptor on hepatocytes, thus increasing the otherwise physiologic hepatic clearance of platelets. (11, 12)

It is not known which of the pathways are responsible for the thrombopenia in the specific ITP patient. A better understanding of these mechanisms could have clinical implications, such as predicting the effectivity of splenectomy as treatment for refractory ITP.

Imaging techniques using nuclear agents, such as the 111-indium, are able to visualize and quantify platelet sequestration in splenic, hepatic or mixed patterns. Studies moreover suggest that when a splenic sequestration is found, a splenectomy shows higher success rates compared to a mixed or hepatic pattern. (13, 14)

In ITP, a possible association between platelet autoantibody specificity and sequestration site of platelets has not been studied previously. (15, 16) One of the reasons for this lack of data might be that the auto antibody detection in ITP has only recently become sensitive and specific enough. (8, 17-20)

This study aims to investigate the association between GP autoantibodies with anti-GPIIb/IIIa, GPIb/IX or GPV specificity and sequestration site of platelets in a cohort of treatment relapse and refractory ITP patients. Secondary objectives include investigating the role of platelet clearance rate calculated for both sequestration sites, and in relation to the presence or absence of platelet auto-antibodies.

Methods

Design and patients

A retrospective cohort study included all adult ITP patients who had an 111-Indium labeled platelet sequestration study in the Netherlands between 2014 and 2018. A sequestration study was performed if an ITP patient had an indication for successive therapy, and splenectomy is considered as one of the therapeutic options. Therefore, the results of the sequestration studies were not used as a direct pre-operative screening in this cohort.

Antibody measurements

Blood samples from all patients were taken at the first day of the indium labeled sequestration study as a routine diagnostic procedure and were sent to Sanquin Diagnostic Services in Amsterdam, the Netherlands for auto-antibody detection. Patient platelets, platelet eluates and sera were tested, within 24 hours after sampling, with the direct and indirect platelet immunofluorescence test (PIFT) as described by von dem Borne et al (7, 21) for the presence of platelet-associated and free circulating autoantibodies of the IgG- and IgM-class. Furthermore a modified direct and indirect MAIPA was used, as described by Kiefel et al (22), to investigate the presence of GPIIb/IIIa, GPIb/IX and GPV-associated autoantibodies of the IgG-class. (7, 8) The direct MAIPA was used in our primary analyses with a cut-off for positivity of AU=0.130.

111-Indium labeled sequestration study

The 111-Indium labeled sequestration study is performed in accordance with the recommendations of The International Committee for Standardization in Hematology Panel on Diagnostic Application of Radionuclides. (23) In short, 50ml whole blood is derived from the patient and labelled with 111-Indium tropolone. Autologous 111-Indium labeled platelets are re-injected in ITP patients. At t=30 minutes, 3, 24, 48 hours after reinjection of labelled platelets, new blood samples are taken for platelet survival studies. Gamma camera used for this study is 111-In NMG, with peaks at 172 and 247 keV. Dynamic series are made for 25 minutes at t=30 minutes. At t=30min, 3, 24 and 48h, static series are made for 5 minutes. At all series both posterior and anterior scans are made. Categorization of

platelet sequestration patterns was adopted from Najean et al (9) where scan outcome is a ratio between liver and spleen in percentages at 30 minutes, 24 hours and 48 hours after reinjection of labeled platelets. Percentages of sequestration in spleen and liver add up to 100%. In the clinical setting, the sequestration is categorized in splenic, mixed and hepatic pattern based on the splenic: liver ratio (S:L ratio). S:L ratio >1.4 is described as a splenic sequestration pattern, 0.8 < 1.4 as mixed pattern and < 0.8 as hepatic platelet sequestration pattern. The percentage of splenic sequestration (0-100 %) is used as a continuous variable in our primary analyses. The clearance rate is measured using the loss of radio-activity in the intervals between 0-24h and 24-48h. At time point 0h the radio-activity is 100 percent. Loss of radio-activity is used as a proxy for platelet clearance rate. The higher the loss radio-activity, the higher the clearance rate is hypothesized to be. The Haga Teaching Hospital in The Hague, the Netherlands, performed all 111-indium labeled sequestration studies.

Data and statistics

This study was approved by the central medical ethical review board in the Netherlands and by the local review board of the Haga Teaching Hospital in the Hague. Informed consent was waived due to the retrospective design of the study. Data on patient characteristics, antibody measurements and 111-Indium labeled sequestration study were collected from the electronic patient files. Statistics were performed in IBM SPSS version 25. Patient characteristics were analyzed in a descriptive manner, and stratified for antibody presence. We used linear regression models to investigate the associations between antibody presence and sequestration site. Age, sex, platelet count and treatment lines prior to scan were used as covariates in the multivariate models.

Results

Baseline characteristics

The baseline (**table I**) shows the cohort consisting of 74 ITP patients of whom 66% are females. Mean age at the time of the 111-Indium labeled sequestration study was 43 (±17) years; most patients (74%) were younger than 60 years. Mean age at the time of diagnosis of ITP was 36 (±18) years. Median platelet count at the time of the scan was 52 x 109/L (IQR 32 – 116), 22% of all patients had platelet counts lower than 30 x 109/L. The majority of the patients (69%) had successful medicinal treatment for their ITP at the time of scan and therefore had relative high platelet counts. Bleeding scores were low with only 7% of patients having a WHO bleeding score ≥3 from the time of diagnosis. 35% of the patients received 1-2 lines of therapy, 53% received 3 or 4 lines therapy and 13% received 5 or more lines of therapy prior to the scan.

Table I – Baseline characteristics

	All (n=74)	Anti-glycoprotein positive* (n=30)	Anti-glycoprotein negative* (n=27)
Age at scan, *years*	43 ±17	43 ±17	44 ±17
<60 years	74%	73%	74%
> 60 years	22%	27%	19%
Age at Dx ITP, *years*	36 ±18	39±17	34±20
Gender, % female	66%	70%	52%
Platelet count, x109/L	52 [IQR 32, 116]	67[IQR 39,140]	46[IQR 33,84]
Comorbidities >1	31%	33%	33%
Highest bleeding score			
.WHO 1	30%	23%	37%
.WHO 2	49%	60%	44%
.WHO 3 or higher	7%	0%	11%
Treatment history			
.Initial wait & see	10%	10%	15%
.Corticosteroids	84%	93%	82%
.Rituximab	23%	33%	15%
.IVIG	35%	30%	52%
.TPO-ra	43%	43%	44%
Number of treatment lines in history			
.1-2	35%	27%	26%
.3-4	53%	53%	44%
.5 or more	13%	13%	15%
Treatment strategy at time of scan			
.No treatment	43%	40%	44%
.Corticosteroid alone	27%	33%	26%
.TPO-ra alone	19%	17%	15%
.other treatments**	11%	10%	15%

17/74 (23%) patients had no antibody testing

*Antibodies were considered present when the direct MAIPA was 0.130 or higher.

** other treatments consisted of either IVIG alone (n=1 patient) or a combination of corticosteroid & IVIG (n=4), combination of corticosteroid and TPO-ra (n=3)

Comorbidities was defined as relevant systemic comorbidity in one of the systems: heart disease, lung disease, kidney failure, malignancy or auto-immune disease.

TPO-RA: Thrombopoietin receptor agonist.

IVIG: Intravenous Immune globulin. WHO bleeding scale: 0=No bleeding, 1 = Petechiae, 2=Mild blood loss, 3=Gross blood loss, and 4=Debilitating

Association between antibodies and sequestration pattern

Of the 57 tested patients 30 (53%) showed one or more GP antibody specificity. Forty-seven percent of the patients had a splenic sequestration pattern, 29% mixed and 25% an hepatic pattern. **Table IIa** shows the number of patients with a splenic, mixed and hepatic sequestration pattern, stratified by this presence of autoantibodies. In the patients where autoantibodies were present, 47% had a splenic pattern, 30% mixed and 23% a hepatic

pattern. In the patients where no autoantibodies were found, 41% had a splenic pattern, 33% mixed and 26% a hepatic pattern. The various sequestration patterns were not different if the full cohort was compared with that of the patients tested for platelet autoantibodies (data not shown), or in the subgroups of patients with or without platelet autoantibodies.

Table IIa – Sequestration pattern at 48h stratified by antibody

	Anti-glycoprotein positive* (n=30)	Anti-glycoprotein negative* (n=27)
Splenic pattern	14 (47%)	11 (41%)
Mixed pattern	9 (30%)	9 (33%)
Hepatic pattern	7 (23%)	7 (26%)

*Antibodies were considered present when the direct MAIPA was 0.130 or higher.

Table IIb shows the presence of autoantibodies stratified by sequestration pattern. Patients with a splenic pattern showed a higher percentage of anti-GPIIb/IIIa and GPV compared to patients with a hepatic pattern (71% vs 57% and 71% vs 29% respectively). The opposite was found for anti-GPIb/IX, where patients with a hepatic pattern show a percentage of 71% vs 29% in splenic pattern group.

Table IIb – Type of antibodies in patients where antibodies were found stratified by sequestration pattern at 48h

	Anti-GPIIb/IIIa level* n (%)	Anti-GPIb/IX level* n (%)	Anti-GPV level* n (%)
Splenic pattern *n=14*	10 (71%)	6 (43%)	10 (71%)
Mixed pattern *n=9*	5 (56%)	4 (44%)	7 (78%)
Hepatic pattern *n=7*	4 (57%)	6 (86%)	2 (29%)

*Patients can have more than 1 antibody present, thus the groups are not mutually exclusive.

Table III shows the association between the sequestration site and antibody type in crude and multivariate models. The univariable association between platelet sequestration pattern and anti-GPV antibodies was 0.011 (95% CI 0.001 - 0.021), p=0.034. The coefficient for anti-GPIIb/IIIa and anti-GPIb/IX antibodies was 0.003 (95% CI -0.009-0.015) p=0.635 and 0.001 (95% CI -0.012-0.014) p=0.853 respectively. The results from the multivariate models, including age, sex, platelet count and treatments did not show major differences compared to the crude associations.

Table III – Linear regression for the association between indium labeled platelet scan and detected antibodies with direct MAIPA

	Anti-GPIIb/IIIa level *ß (95%-CI),* *p value*	Anti-GPIb/IX level *ß (95%-CI),* *p value*	Anti-GPV level *ß (95%-CI),* *p value*
Splenic sequestration (%)	0.003	0.001	0.011*
at 48h	(-0.009 – 0.015)	(-0.012 – 0.014)	(0.001 – 0.021)
crude effect	p=0.635	p=0.853	p=0.034
Splenic sequestration (%) pattern	0.002	0.000	0.010
at 24h	(-0.010 – 0.015)	(-0.013 – 0.014)	(0.000 – 0.021)
crude effect	p=0.723	p=0.967	p=0.058
Splenic sequestration (%) pattern	-0.001	0.003	0.014*
at 30m	(-0.017 – 0.014)	(-0.014 – 0.019)	(0.001 – 0.026)
crude effect	p=0.890	p=0.731	p=0.034
Multivariate models			
Splenic sequestration (%) pattern	0.003	0.003	0.010
at 48h	(-0.009-0.016)	(-0.011-0.016)	(-0.001-0.021)
+ age	p=0.604	p=0.697	p=0.071
Splenic sequestration (%) pattern	0.002	0.002	0.011*
at 48h	(-0.009 -0.014)	(-0.010 -0.015)	(0.001-0.022)
+ sex	p=0.672	p=0.720	p=0.031
Splenic sequestration (%) pattern	0.002	0.001	0.011*
at 48h	(-0.010 – 0.015)	(-0.012 – 0.015)	(0.001 – 0.022)
+ platelet count	p=0.717	p=0.831	p=0.040
Splenic sequestration (%) pattern	0.001	0.001	0.010
at 48h	(-0.012 – 0.014)	(-0.010 – 0.011)	(-0.001 – 0.022)
+ treatment	p= 0.922	p= 0.930	p=0.064

Association tested with univariable and multivariable linear regression models. Antibody variables were log transformed for normalization. MAIPA: Monoclonal Antibody-specific Immobilization of Platelet Antigen.

Association between sequestration site and clearance rate

We found an association between a splenic sequestration and a faster clearance rate in patients where GP antibodies were present (beta coefficient 0.186, SE 0.066, p=0.009). We did not find an association between the sequestration site and the overall clearance rate (beta coefficient 0.046, SE 0.029, p=0.119). **Table SII** (supplementary materials) shows the results of the regression analyses for platelet sequestration pattern at different time points (30 min, 24 hours, 48 hours) and platelet clearance rate.

Discussion

The aim of this study was to investigate if GP auto-antibodies are associated with a specific sequestration pattern or clearance rate of Indium labelled platelets in relapse/refractory ITP patients. This study showed no association between the overall presence of GP-antibodies, and a splenic sequestration pattern. However, within the cohort of GP-antibody positive patients, this association was found in the presence of GPV autoantibodies. No significant associations were found between anti-GPIIb/IIIa or GPIb/IX and sequestration pattern. The presence of anti-GPIb/IX seemed to be more pronounced in patients with a hepatic sequestration pattern, but was not significant. Additionally, in the patients with GP antibodies, an association was observed between higher clearance rate and splenic sequestration. In patients without GP antibodies no associations were found between clearance rate and a specific sequestration pattern.

Antibodies in ITP

Although the pathophysiology of ITP is not fully elucidated, autoantibodies are regarded to play an important role in the disease. (24) Autoantibodies directed against GPIIb/IIIa, GPIb/IX and GPV are the most frequently found in immune thrombocytopenia, and held to be responsible for increased platelet clearance while possibly also for impaired platelet production and platelet function. (7, 8, 25-28) Complement activation is more and more found to be possible important in these mechanisms. (18, 29) Antibody positivity and load in general might be associated with more severe or refractory ITP, as shown in a study where a significant association between non responsiveness on rituximab treatment and lack of detectable platelet autoantibodies was found. (20, 30) Additionally, different antibody specificities were suggested to induce different mechanisms of platelet destruction and this has been subject of research for the past decades. (31) Studies showed that GPIIb/IIIa antibodies are associated with clearance via Fc-receptor mediated clearance by splenic macrophages (6, 32) whilst GPIb/IX antibodies are associated with Fc-independent hepatic clearance via the Ashwell Morell receptor. (33) Our observations in this cohort of ITP patients are in line with these associations. We found a higher percentage of patients with anti- GPIIb/IIIa (71%) and/or anti-GPV (71%) as compared to patients with anti-GPIb/IX (43%) if there was mainly splenic sequestration. In patients with hepatic sequestration we find a higher number with anti-GPIb/IX (86%) as compared to patients with anti-GPIIb/IIIa (57%) and/or anti-GPV (29%). In regression models only anti-GPV and a splenic sequestration pattern show a significant association. A limitation of our study is the small sample size which makes it underpowered to detect a significant association. Future studies are needed to build further on the associations and effect sizes found in this study.

Interestingly, we did find a significant association between the presence of anti-GPV and a splenic sequestration pattern. Until recently, the role of anti-GPV antibodies in ITP has not been described extensively. GPV is noncovalently linked to the GPIb/IX complex and a major fragment, i.e. almost the entire extracellular part, of GPV is released after platelet activation by thrombin, elastase and calpain. (34) Modderman et al. investigated GPV expression on the platelet membrane and showed that 11,000 intact GPV molecules are present on resting platelets from healthy individuals and that cleavage of the majority of GPV occurs after exposure to thrombin. (34) We therefore assume that in the majority of ITP patients sufficient GPV molecules are present on the platelet membrane for detecting platelet-associated autoantibodies and for autoantibody induced platelet destruction. Recent publications hypothesized that anti-GPV may act in the same way as anti-GPIb/IX antibodies through binding on the GPIb/IX-V complex on the platelet surface. (25) Vollenberg et al. found anti-GPV antibodies in the majority of their autoantibody positive patient population, with comparable prevalence to our cohort. GPV antibodies showed induction of platelet destruction in vivo (in NOD/SCID mouse models) and in vitro, irrespective of their avidity. Overall clearance of platelets is higher when patients have also GPV antibodies either 1) by increasing the overall IgG load, leading to overall more platelet clearance or 2) specific functional effects of anti-GPV with changes in platelet reactivity.(25, 35) Our study suggests that GPV antibodies opsonize platelets for Fc-mediated clearance in the spleen, and might have a distinct clearance pathway from other antibody specificities in ITP.

Glycoprotein Ib/IX on the surface is known to be an important aspect in platelet physiology; especially for its interaction with von Willebrand factor (VWF). However, more physiological roles are discovered with GPIb/IX, as GPIb/IX activation by ligand binding under shear stress. (36) One study suggests in animal model studies that specific antibodies against GPIbα N-terminus cause platelet clearance in the liver, and this may be associated with desialysation as part of the aging-process of platelets, when aged platelets are cleared by the liver. (11, 33) In our cohort we see relatively higher percentages of anti-GPIb/IX in patients with a hepatic sequestration pattern. This may incline a confirmation of the studies by Li et al. in a first in-vivo human study. (33)

Rate of clearance

In this study we also examined the rate of clearance in association with sequestration site, and found an increased rate of platelet clearance in antibody-positive patients with splenic sequestration. Antibody positivity and higher load may be leading to faster clearance, and faster clearance may to some extent be seen as a proxy for disease severity in ITP. This is in line with what Al-Samkari et al found. (20) Their study showed that antibody positivity and load are associated with more disease severity. (20) The impact of this finding is still

unknown and should be investigated further together with other pathways, such the role of T regulatory cells and direct CD8+ mediated cytotoxicity. (37, 38)

Strengths and limitations

The strength of this study is that we for the first time collected data on platelet autoantibodies, autoantibody specificities in relation to sequestration in spleen, liver or both in a group of ITP patients. Limitations were that although we included 74 patients, platelet antibodies were tested in 57 patients. Due to the lack of previous data on this subject no power calculations could be performed, thus creating the possibility of a low power to detect associations, especially when looking at subgroups of patients. This study provides the opportunity for future researchers to validate these new insights and study possible clinical implications for treatment choice. For generalizability we included all patients who had a scintigraphy in our hospital.

Second, it is unknown which variables may confound the associations, partly due to the heterogeneous nature of ITP. In this study we included the most used variables, such as age, sex, platelet count and treatment history.

Third, in this study we did not measure the IgA and IgM, or IgG subclasses. It has been suggested that abnormalities in levels of IgA and IgM lead to more treatment-resistant ITP. (39). Furthermore, this cohort lacked data on the presence of anti-GPIa/IIa, anti-GPIV and anti-GPVI which has been reported in some patients with ITP and may impact the site of platelet destruction, but is not measured in the direct MAIPA that was used in this study. (7, 40, 41)

Fourth, the percentage of platelet autoantibody positivity in our cohort was lower than expected, given the sensitivity of the direct MAIPA. The sensitivity of MAIPA is 81% and the specificity of MAIPA is 98% for clinical diagnosis of ITP. (7) In the investigated ITP cohort platelet autoantibodies were detected in only 53% of the patients, which might be due to the selection of relapsed/refractory patients, but also some patients were treated successfully.

Fifth, this cohort has not yet follow-up data up on the clinical outcome and splenectomy was performed. A longer follow up is needed and will be insightful for the clinical implications of these findings.

Conclusion and future perspectives

In this study we found in relapse/refractory ITP patients an association between splenic sequestration of platelets and presence of GPV antibodies. Furthermore, our data suggest that patients with a hepatic pattern showed more often the presence of GPIb/IX antibodies. Moreover, this study found an association between clearance rate and an splenic sequestration pattern in in anti-GP positive patients. Future studies are needed to validate

the associations and investigate the clinical implications. This study adds to the knowledge needed to better understand and individualize diagnosis and treatment of ITP.

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Supplementary tables and files

Table SI – Distribution of antibody specificities & combinations of antibodies using direct MAIPA

Anti-GP antibodies	n (%)
IIbIIIa only	9 (16%)
IbIX only	1 (2%)
V only	5 (9%)
IIbIIIa & V combination	2 (4%)
IbIX & V combination	3 (5%)
IIbIIIa & IbIX combination	1 (2%)
IIbIIIa & IbIX & V combination	9 (16%)
Antibody positive	30 (54%)
Antibody negative	27 (47%)
Total	57 (100%)

Table SII – Linear regression for the association between sequestration pattern and clearance rate

	Platelet clearance rate		
	Overall *β (SE),* *p value*	Anti-GP pos* *β (SE),* *p value*	Anti-GP neg *β (SE),* *p value*
Splenic sequestration (%) pattern	0.046 (0.029)	0.186* (0.066)	-0.052 (0.118)
at 48 hours	p=0.119	p=0.009	p=0.664
Splenic sequestration (%) pattern	0.038 (0.025)	0.196* (0.069)	-0.037 (0.123)
at 24 hours	p=0.133	p=0.008	p=0.765
Splenic sequestration (%) pattern	0.016 (0.014)	0.264* (0.084)	-0.037 (0.146)
at 30 min	p=0.273	p=0.004	p=0.804

*Antibodies were considered positive when the direct MAIPA was 0.130 or higher.