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Pathogenesis of immune thrombocytopenia in common variable immunodeficiency

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ABSTRACT

Immune Thrombocitopenic Purpura (ITP) is an autoimmune disease characterized by antibody-mediated platelet destruction and variable reduced platelet production. Besides antibody-mediated platelet destruction, new pathogenic mechanisms have been reported to be involved in reducing platelet count. Among these, desialylation is one of the most recent and innovative mechanisms that has been found to be implied, at least in part, in non-antibody mediated platelet clearance.

Common Variable Immunodeficiency (CVID) is the most common Primary Immunodeficiency seen in clinical practice. About 25–30% of CVID patients are affected by autoimmune manifestation, among which ITP is the most common. Little is know about pathophysiological mechanisms that lead to ITP in CVID.

Given the poor antibody production typical of CVID patients, we aimed at verifying whether platelet desialylation could be responsible for CVID associated thrombocytopenia.

According to our results, we may suggest that in CVID patients, ITP is due to a decreased bone marrow platelets production, rather than an increased peripheral platelet destruction, which is more common in patients with primary ITP. An increased platelet desialylation does not appear to be implicated in the thrombocytopenia secondary to CVID, while it is implicated in the pathogenesis of primary ITP. Nevertheless an intriguing aspect has emerged from this study: regardless the presence of thrombocytopenia, the majority of CVID patients present a double platelet population as far as desialylation concerns, whilst no one of the healthy donors and of the patients with primary ITP shows a similar characteristic.

1. Introduction

Immune Thrombocitopenic Purpura (ITP) is an acquired immune-mediated disorder characterized by platelet destruction, possibly associated with a decreased platelet production. The pathogenesis of ITP is poorly understood [1]. The incidence of primary ITP range between 1.9 and 6.4 per 10^5/year in children, and 3.3 per 10^5/year in adults [2].

According to the 2009 IWG (International Working Group) definition, primary ITP is an autoimmune disorder characterized by isolated thrombocytopenia (peripheral blood platelet count $< 100 \times 10^9$/L) without other causes of thrombocytopenia. Consequently, all other forms of immune-mediated thrombocytopenia are secondary ITP [3]. Indeed many clinical conditions can cause secondary ITP, such as rheumatic diseases like antiphospholipid antibody syndrome or systemic lupus erythematous, infections, hematological diseases, liver diseases, drugs and immunodeficiencies (both primary and secondary immunodeficiences) [4].

The diagnosis of ITP is a diagnosis of exclusion and no abnormalities should be found in the blood count, except for the thrombocytopenia. A diagnosis of ITP is made when the history, physical examination, complete blood count, and examination of the peripheral blood smear do not suggest other causes of thrombocytopenia. Bone marrow examination (both bone marrow aspirate and biopsy) is recommended only in patients older than 60 years of age or in those with systemic signs and symptoms suggesting a malignant condition. Routine evaluation for Helicobacter pylori, Human Immunodeficiency virus (HIV) and Hepatitis C virus (HCV) infection in adult patients is recommended. Assays for antibodies to specific platelet glycoproteins are not recommended, given their poor sensibility and specificity [4]. So there is no “gold standard” test that can reliably establish the diagnosis.

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Response to ITP-specific therapy, such as intravenous immunoglobulin (IVIg) and intravenous anti-D, is supportive of the diagnosis, but a response does not exclude secondary ITP.

ITP is classified by duration into newly diagnosed, persistent (3–12 months duration) and chronic (≥12 months duration). While ITP in adults typically has an insidious onset with no previous viral infection or other illnesses and usually has a chronic course, ITP in children is usually of short duration with at least two-thirds recovering spontaneously within 6 months [4].

Signs and symptoms vary widely. Most patients affected by ITP are completely asymptomatic or show minimal signs of bleeding, such as sporadic bruises or epistaxis. Few patients present severe episodes of bleeding, generally including gastrointestinal bleeding, intracranial bleedings and extensive cutaneous or mucosal bleedings. Interestingly, the severity of thrombocytopenia does not correlate with the bleeding risk; instead, age seems to be the only factor that correlates with the bleeding risk [4]. In clinical practice it is not rare to see patients with very low platelet count (1000–2000 plt/μL) who never experienced bleeding.

2. ITP pathogenesis

Like for most of immunomediated diseases, the pathogenesis of ITP is still unclear. According to recent evidences, primary ITP is a multifactorial disease, in which both genetic and environmental factors play a key role, but with different weight among different individuals.

Among environmental factors, infections are important triggers for ITP, especially in children. Potentially, any infection may initiate the events that lead to the onset of an acute ITP, but some viruses such as *Varicella zoster virus* (VZV) and *Cytomegalovirus* (CMV) are known to be typically associated to ITP; also the MMR Vaccine has been associated to the onset of ITP [5].

Other infectious agents known to be associated to ITP are *H. pylori*, HIV and HCV. Different mechanisms may participate to the initiation of the autoimmune process triggered by infections. Molecular mimicry between infectious agents derived epitopes and platelet glycoproteins has been clearly demonstrated: there are sequence homology between epitopes of the GP120 of HIV and the core envelope 1 protein of HCV and the platelet glycoprotein GPIIb/IIIa. Similarities have also been observed between the sequences of different viral proteins derived from *Herpex simplex virus*, VZV, *Epstein-Barr virus*, CMV and the GPIIb/IIIa. Interestingly, some peptides derived from these proteins are recognized by antiplatelet antibodies in vitro.

Other mechanisms are also triggered by infections: *H. pylori* decreases the expression of the inhibitory receptor FcγRIIb on monocytes, CMV and HIV inhibit megakaryopoiesis and chronic HCV infection induces circulating immune complexes that non-specifically bind to platelets, thus accelerating their clearance [1].

Many genes polymorphisms are described as implicated in the onset of ITP, such as polymorphism F158V of CD16 [6] or polymorphisms of FcγRIIA and FcγRIIIA [7]. More recently, variations in the level of some microRNAs, non-coding RNAs that regulate genes at post-transcriptional level, have been reported in ITP. These variations of microRNAs lead to dysregulation of cytokines involved in the immune response, such as INF-γ, IL-21, IL-18 or TGF-β.

2.1. Effector mechanisms of ITP

In 1951 Dr. W. Harrington infused himself with plasma from an ITP patient and developed transient thrombocytopenia [8]. For many years, it has been thought that ITP was merely due to the presence in the serum of autoantibodies that bind antigens on the surface of platelets and mediate their destruction in the reticuloendothelial system. In the last years, our understanding of the pathophysiology of ITP has significantly improved. Thrombocytopenia in ITP is due to different mechanisms, such as platelet destruction by pathologic antiplatelet antibodies, altered desialylation process, impaired megakaryocytopoiesis, and direct T-cell–mediated destruction of platelets. All these pathologic mechanisms play different roles in each patient [1].

2.2. Platelets desialylation

It is well known that only half of the patients affected by primary ITP have antiplatelet antibodies in their sera; moreover, 15–20% of patients do not respond to treatments targeting the classical IgG-opsonizing mechanisms, such as IVIG [1]. Noteworthy, in the latest guidelines of the American Society of Hematology for the treatment of ITP, the role of IVIG has been extensively scaled down [9].

Over the past decades, platelet desialylation has been shown to be responsible for platelet clearance in many contexts, such as destruction of chilled platelets, clearance of senescent platelets, free radicals and infection-related thrombocytopenia [10]. Recently, desialylation has been proposed to be partially responsible for platelets clearance in some ITP patients.

Sialic acid is a natural sugar acid compound that is widely present across organisms. The ends of glycoprotein chains on platelet membranes are all covered by sialic acid to protect them from being destroyed. Sialidases, also known as neuraminidases, are sialic acid-releasing exoglycosidases that catalyse the removal of terminal sialic acids from sialosides and sialoglycoconjugates in nature. Sialidase widely exists in viruses, bacteria, and mammalian cells. Additionally, resting platelets contain an internal pool of sialidases which are released upon activation by any cause or after a pathogenic bacterium-released new sialidase is introduced into the serum. The endogenous and exogenous sialidases hydrolyse terminal sialic acid moieties from platelet glycoproteins. Desialylation leads to the exposure of β-galactose residues on platelets that becomes “desialylated” platelets [11].

According to this model, desialylated platelets are recognized by a specific receptor expressed on the surface of hepatocytes called Ashwell-Morell Receptor (AMR), which is responsible for the platelet phagocytosis by hepatocytes and the subsequent stimulation of thrombopoietin (TPO) production. TPO enter the circulation and stimulates bone marrow production of new platelets; there is also a mechanism through which an increased platelet desialylation corresponds to an increased TPO liver production and an increased stimulus to bone marrow platelet production [12] (Fig. 1).

The AMR is a transmembrane heteroligomeric glycoprotein complex composed of ASGPR1 (CLEC4H1, HL-1) and ASGPR2 (CLEC4H2, HL-2) subunits, which are highly conserved among mammalian species [12].

It has already been reported that ITP patients generally present higher platelet desialylation levels compared to healthy people [10,13,14].

Qiu et al. showed how CD8+ T cells are able to induce platelet desialylation, since the patients whose CD8+ T cells displayed a cytotoxic activity against platelet in vitro, were the ones who also displayed the highest levels of platelet desialylation. The same authors proposed that CD8+ T cells induce platelet desialylation through the translocation of the sialidase Neu1 from the inner granules to the surface of the platelets, resulting in an increased sialic acid cleavage [10].

Li et al. showed how Ig-mediated mechanisms and non Ig-mediated mechanisms of platelet clearance could be intimately linked to each other, since anti-GpIbα antibodies (but not anti-GpIIbIIIa) are able to induce Neu1 translocation on the platelet membrane and consequently to increase desialylation levels; moreover there could be a linkage even between platelet activation and desialylation, synergizing in determining platelet clearance [13]. Similar results were shown by Marini

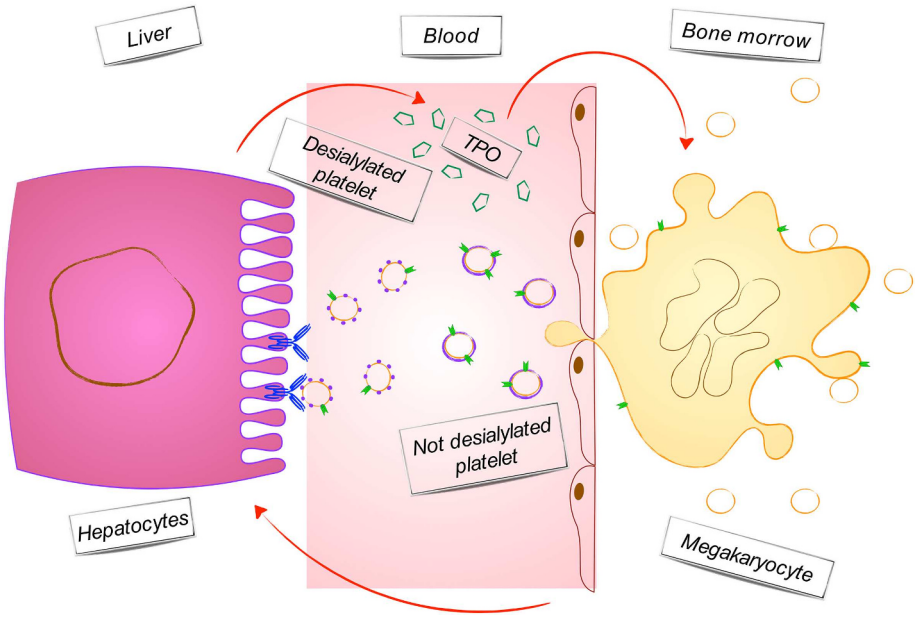

Fig. 1. Schematic representation of recognition of desialylated platelets by the specific receptor (Ashwell-Morell Receptor) expressed on the surface of hepatocytes, which is responsible for the platelet phagocytosis by hepatocytes and the subsequent stimulation of thrombopoietin (TPO) production. TPO enter the circulation and stimulates bone marrow production of new platelets.

I. et al., furthermore demonstrating that desialylating antibodies are also able to induce platelet disfunction (reduced adhesion) and impaired in vitro thrombopoiesis [15].

An increased platelet desialylation could be predictive of treatment response in patient with ITP, since patients exhibiting higher levels of platelet desialylation tend to be less responsive to first line treatments such as corticosteroids or IVIG [14,16].

The efficacy of oseltamivir (a sialidase inhibitor primary used in the treatment of Influenza virus infection) in raising platelet count has been reported [16,17].

Based on these findings it is becoming increasingly clear that ITP can no longer be considered a homogeneous disease.

3. Common variable immunodeficiency

Common variable immunodeficiency (CVID) is considered a heterogeneous group of primary immune deficiency diseases characterized by reduced serum levels of IgG, IgA, and/or IgM, with decreased antibody production and impaired antibody response to both polysaccharide and protein antigens [18,19]. CVID is considered the most commonly encountered and clinically relevant primary immunodeficiency in adults and though the majority of patients are diagnosed between the age of 20 and 40 years, at least another 20% are diagnosed during childhood (> 2 years) or adolescence. The typical defect of CVID is the failure of Blymphocytes to differentiate into switched memory B cells and into plasma cells [20]. Several abnormalities of T cells have also been described in CVID including oligoclonal expansion of CD8+ T cells, decreased numbers of CD4+ T cells, T cells activation defects, apoptosis and anergy, impaired prolipheration in response to mitogens, disruption of CD4+ and CD8+ TCR repertoire, reduction of CD31+ recent thymic emigrants CD4+ T cells, reduced expression of CD40L on activated T cells, and low levels of IL-2 mRNA. Moreover, T lymphocytes show an impaired secretion of several soluble mediators, which may contribute to the B cell differentiation failure. Finally, the frequency and suppressive function of regulatory T cells (Tregs) are altered in CVID patients. The expression of FOXP3 protein and also levels of inhibitory cytokines such as IL-10 are diminished in CVID [21]. The decrease of Treg cells is more pronounced in CVID patients with a severe decrease of switched memory B cells and expansion of $CD21^{low}$ B cells [22]. As far as the innate immune system concerns, some reports have shown that dendritic cells present a severely altered differentiation, maturation, function, and reduced levels of co-stimulatory molecules that are critical for T cell activation [23]. Moreover, a peripheral decreased number of natural killer cells and monocytes alterations directly correlating with T cell activation markers and with B cell imbalances have been reported in CVID [24,25].

The typical symptoms of CVID are infections that may involve respiratory, gastrointestinal, and genitourinary tracts [21]. Autoimmune manifestations are not uncommon in patients with CVID and the association between autoimmune disease and CVID is well recognized, since autoimmune diseases occur in approximately 20–30% of patients with CVID. The coexistence of immunodeficiency and autoimmunity appears paradoxical; while antibody production in response to pathogens and vaccines is severely impaired or even lacking, the generation of autoantibodies might, at the same time, be excessive. Moreover, autoreactive B and T cells can be detected in patients with CVID, even if specific response to antigens is impaired. On the other hand, considering that both innate and adaptive immune response abnormalities occur in CVID, such as expansion of abnormal B cell clones with altered trafficking and effector functions, altered cytokine expression and signaling, it is easy to understand the mechanisms that lead to a breakdown of self-tolerance in CVID [26].

The most common autoimmune hematological abnormalities found in CVID patients is ITP, that recur approximately in 14% of patients, and anemia or neutropenia (7% and 1% respectively). These complications are frequently associated with splenomegaly; however, hypersplenism is unable to completely explain this association and the pathophysiological link remains unclear. It is important to underline that in more than 60% of CVID patients, the diagnosis of cytopenia may precede by many years the detection of hypogammaglobulinemia [18,27].

The mechanisms that lead to development of ITP in CVID remain unclear.

It is unlikely that ITP in CVID might be due to anti-platelet antibodies since the typical CVID defect is the inability of B cells to produce antibody.

Thrombocyte sequestration in splenomegaly can cause ITP. However, splenomegaly is a frequent finding in all CVID patients and

many CVID patients have splenomegaly but not ITP, suggesting that other factors may also contribute to the development of thrombocytopenia.

Of particular relevance is the correlation between an expansion of $CD21^{low}$ B cells, autoimmune disease and splenomegaly [28]. We recently observed that in patients affected by CVID and ITP the number of $CD21^{low}$ B cells is higher than in CVID patients without ITP [26]. The $CD21^{low}$ B cells are a subset of B cells that appear to be in an activated state but at the same time express several inhibitory receptors. They do not proliferate in response to BCR triggering and are also prone to undergo apoptosis. However, these cells are able to respond to other stimuli and interact with other cells such as T lymphocytes subsets and/or innate immune cells. Moreover, $CD21^{low}$ B cells may be enriched in autoreactive clones [29].

In addition, correlations between an expansion of transitional B cells with lymphadenopathy and ITP with reduced plasmablasts, preterminally differentiated plasma-cells, have been identified [28].

As far as T cells subsets concerns, the decreased number of CD4+ naïve T cells and increased number of activated CD4+ T cells are related not only to autoimmune cytopenia but also with splenomegaly and expansion of $CD21^{low}$ B cells [30].

Finally the number of terminally differentiated CD8+ cells is significantly higher in CVID patients with polyclonal lymphoid proliferation and autoimmune cytopenias than in CVID patients without autoimmune manifestations [31].

All these observations report a particular correlation between autoimmune cytopenia and particular cell subsets or clinical features, but the pathophysiological mechanism that leads to autoimmune cytopenia in CVID remain elusive and unclear.

Since platelet desialylation is a non Ig-mediated mechanism that sheds a new light into platelet clearance, we have evaluated platelet desialylation in CVID patients with ITP in order to better clarify one of the mechanisms that may contribute to develop ITP these patients.

4. Materials and methods

4.1. Patients

In the present study we enrolled 40 subjects, divided into 4 groups: 10 healthy donors (HDs group), 10 CVID patients who never presented thrombocytopenia (CVID group), 10 CVID patients with past or actual thrombocytopenia (CVID + ITP group) and 10 patients affected by primary ITP (ITP group). All patients affected by CVID attended the Unit of Clinical Immunology at the University Hospital in Verona, Italy and fulfilled the 2014 ESID diagnostic criteria, whereas primary ITP patients attended the Unit of Hematology of the same hospital.

All CVID patients were treated with regular monthly infusions of immunoglobulins (IG: 0.4 g/kg); in particular 13 patients with intravenous (IV) IG and 7 with subcutaneous (SC) administration. 5 out of 10 ITP patients were on treatment with corticosteroids or other immunosuppressive drugs at the time of blood sampling.

For the HDs group we selected 10 subjects not affected by thrombocytopenia, autoimmune diseases or other sistemic diseases.

For each subject 3 EDTA K2 peripheral blood samples were obtained.

A written informed consent was obtained from all the participants to the study and the study was approved by the local ethical committee.

4.2. Reticulated platelets

The reticulated platelet count was obtained in the Central Laboratory of the University Hospital in Verona, using Sysmex XN-9000 (Sysmex Corporation, Kobe, Japan) automated full blood cell count, able to identify the immature platelet fraction (IPF).

4.3. Plasmatic TPO assay

For each subject we evaluated plasmatic TPO level using a commercial enzyme-linked immunosorbent assay (ELISA) kit (TPO Quantikine ELISA, R&D Systems, Abingdom UK), following the manufacturer' instructions.

4.4. Platelet desialylation

Whole blood was collected in EDTA coated tubes from each subjects and platelet rich plasma (PRP) was obtained by centrifugation at 200*g* for 10′.

Five hundred μL of PRP were then diluted with 1 mL of phosphate buffer saline (PBS) and centrifuged at 850*g* for 5′, and platelets pellet resuspended in 500 μL of PBS. Three μL of this suspension were resuspended in 100 μL of PBS and incubated with FITC labelled antibody CD42a (BD Bioscience, San Jose, CA, USA) for 20′ at 4 °C. Labelled platelets were therefore analysed by flow cytometry to determine the gate for evaluation of platelet desialylation.

Six μL of PRP in 200 μL of PBS was labelled with 2 μL of FITC labelled *Ricinus communis* Agglutinin 1 (Vector Laboratories, Burlingame, CA, USA), incubated for 20′ and then analysed by flow cytometry using the gate previously determined. All analysis were performed using FACScanto II (BD Bioscience).

4.5. Statistical analysis

All statistical analysis were performed using GraphPad Prism version 5 (GraphPad Softwere Inc., La Jolla, CA, USA). Data with a normal distribution were analysed with Student' *t*-test and were expressed as a mean +/− standard deviation. A difference between groups with a $p < 0.05$ was considered statistically significant.

5. Results

In this study we used the percentage of reticulated platelets of the total platelet count as an index of bone marrow ability to produce new platelets. According to this parameter, we found a statistical difference between the ITP group and the other three groups (Fig. 2).

Even if there is not a precise cut-off defining the normal range of reticulated platelets, we found that 8 out 10 patients of the CVID + ITP group presented a low reticulated platelet count.

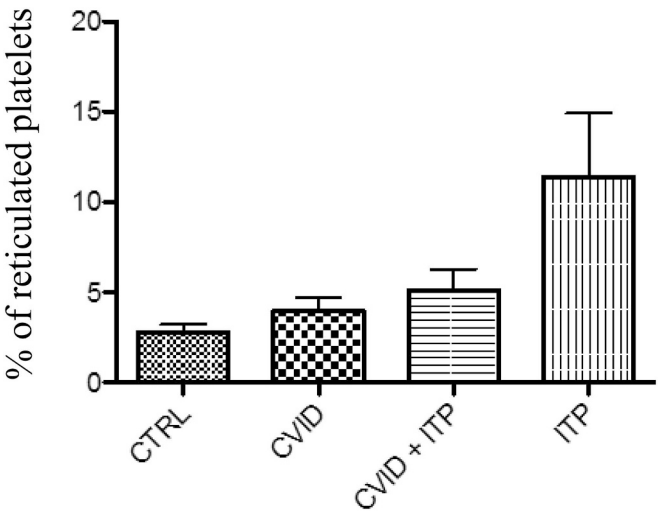

Fig. 2. Percentage of reticulated platelets in the different groups analysed. ITP vs CTRL: $p = 0.0018$; ITP vs CVID: $p = 0.0142$; ITP vs CVID + ITP: $p = 0.0376$. CTRL: healthy donors; CVID: common variable immunodeficiency; ITP: immune thrombocytopenia.

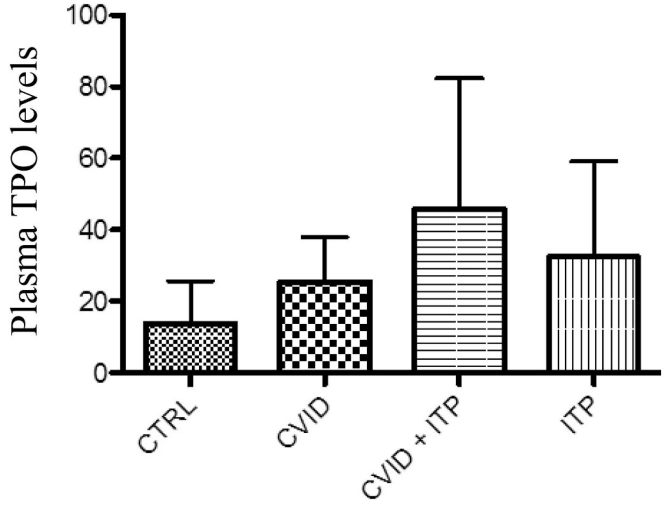

Fig. 3. Plasma Thrombopietin levels (pg/mL) in the different groups analysed. CVID + ITP vs CTRL: p = 0.0086. CTRL: healthy donors; CVID: common variable immunodeficiency; ITP: immune thrombocytopenia.

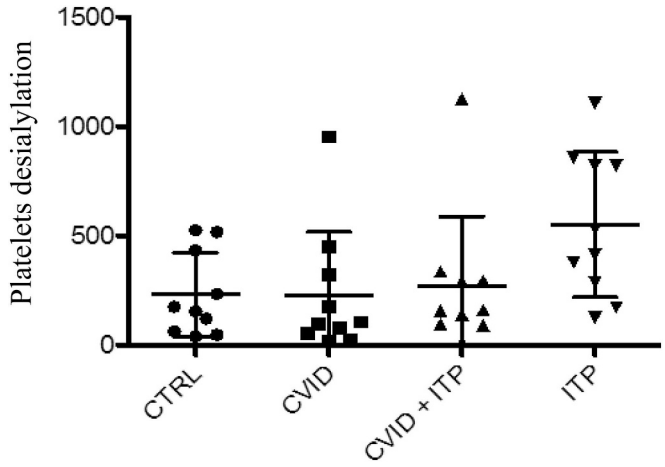

Fig. 4. Platelets desialylation in the different groups analysed. ITP vs CTRL: p = 0.0116; ITP vs CVID: p = 0.0093; ITP vs CVID + ITP: p = 0.0177. CTRL: healthy donors; CVID: common variable immunodeficiency; ITP: immune thrombocytopenia.

With regard to the plasmatic TPO values, all three pathological groups (CVID, CVID + ITP and ITP) displayed significantly higher values compared to the HDs group, with the CVID + ITP group displaying the highest levels (although without significant difference compared to the CVID and the ITP groups) (Fig. 3).

As far as the platelet desalylation concerns, the ITP group presented much higher levels of platelet desialylation compared to the other three groups (Fig. 4); moreover it was the only group in which a linear correlation between the TPO levels and desialylation values was present (Pearson r 0.64, R^2 0.46) (Fig. 5).

Finally, regardless of the absolute RCA-1 MFI value, but looking at the morphology of the fluorescence graph, in 12 out of 20 of the CVID patients (counting both CVID and CVID + ITP groups) it was possible to clearly distinguish a double platelet population, one less and one more desyalilated. This double peak pattern was never found in the subjects of the HDs and ITP groups (Fig. 6).

6. Discussion

Immune thrombocytopenia is the most frequent autoimmune manifestation in CVID patients. Different reports describe how some clinical features such as splenomegaly, lymphoproliferation, granulomatous disease [28], low circulating switch-memory B cells or immunosenescence [32], correlate with the presence of thrombocytopenia but little is known about pathophysiological mechanisms that lead to the development of ITP in CVID patients.

Our results suggest that thrombocytopenia with a low reticulated platelet fraction is due to a reduced medullary production, while thrombocytopenia with a high reticulated platelet fraction is due to an increased peripheral platelet destruction, together with an attempt by the bone marrow to reconstitute a normal platelet level.

According to this, we suggest that thrombocytopenia during CVID is due to a reduced platelet production in the great majority of cases. On the other side, we found that in primary ITP, thrombocytopenia is mostly due to an increased peripheral platelet destruction.

TPO level in the CVID + ITP group is significantly higher compared to healthy donors, suggesting an attempt to stimulate platelet production, but the bone marrow seems somehow unresponsive.

It has been reported that CVID patients' bone marrow presents very frequently T cells infiltrates [33] that could be responsible for most cases of thrombocytopenia, interfering someway with megakaryopoiesis.

The plasmatic TPO levels were significantly higher also in the ITP group than in HDs, but in this case, also the reticulated platelet fraction was increased.

However, in none of the groups a linear correlation between the plasmatic TPO levels and the reticulated platelet levels was found, indicating that the bone marrow response to TPO stimulation is different in different subjects, and it is far too complex to be analysed in such a simple way.

The principal aim of this study was to evaluate the possible role of platelet desialylation in the pathogenesis of thrombocytopenia complicating CVID. According to the data obtained, there is clearly no difference in the RCA-1 MFI values among the HDs group, the CVID group and the CVID + ITP group. On the contrary, the ITP group

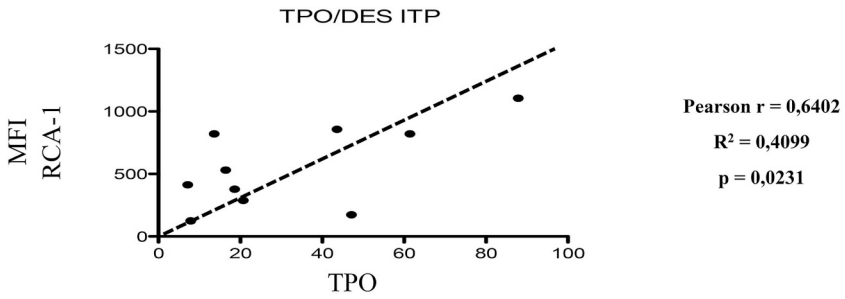

Fig. 5. Correlation between desialylation and TPO levels in the ITP group. TPO: thrombopoietin; ITP: immune thrombocytopenia.

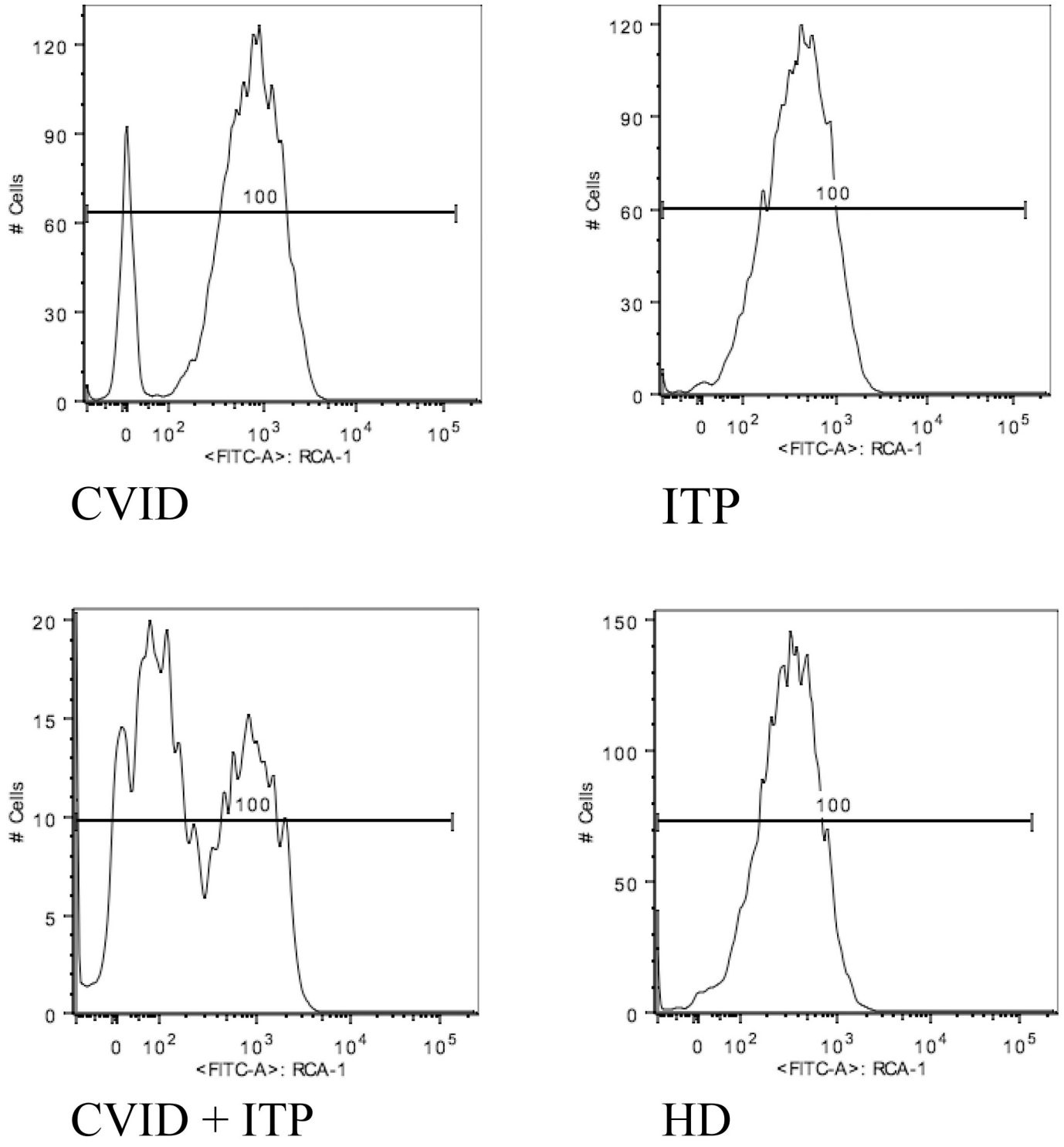

Fig. 6. CVID and CVID + ITP patients present two platelet populations as far as desalylation concerns. This is not present in the CTRL and in ITP groups. CTRL: healthy donors; CVID: common variable immunodeficiency; ITP: immune thrombocytopenia.

presented significantly higher levels of platelet desialylation than the other three groups, strengthening the data already present in literature on the importance of desialylation as biomarker/prognostic tool in identifying the subset of patients refractory to the usual treatments in whom the use of sialidase inhibitors could be beneficial.

In addition, the ITP group was the only one in which there was a linear correlation between platelet desialylation and plasmatic TPO levels, suggesting that increased platelet desialylation stimulates TPO liver production. This finding is in accordance with the mechanism proposed by Grozowsky et al. [12].

The finding that most of the CVID patients, both affected and not affected by thrombocytopenia, presented a double peak pattern in the MFI fluorescence graph is very intriguing and suggest that there are different population of platelets and this aspect may in part explain the predisposition of CVID patients to develop thrombocytopenia.

We are planning to increase the number of subjects enrolled, and to test the role played by CD8+ T cells in platelets desialylation and destruction in CVID patients.

In conclusion, desialylation does not seem to play a role in the pathogenesis of ITP in CVID patients whereas it plays an important mechanism in primary ITP.

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