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Platelet evaluation in immune thrombocytopenia

DOCTORAL THESIS

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Cuprins

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Lista cu lucrările științifice publicate

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2. Particularități în tratamentul trombocitopeniei imune cronice – experiența Clinicii de Hematologie a Spitalului Universitar de Urgență București

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3. Tendinte actuale in ITP- mai mult decat o singura afectiune

Autori Ana Maria Vladareanu, Alina Mititelu

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The fundamental problem

The topic of the scientific research project that based this doctoral thesis refers to the evaluation of platelets in primary immune thrombocytopenia, not necessarily as a direct investigative method, but rather as an analysis of their implication in the serological and clinical picture, as well as in terms of their immune involvement, in this highly heterogeneous condition affecting this category of patients.

It is considered that the primary function of platelets underlies the mechanisms of hemostasis and thrombosis. Considering recent research in the field, the platelet's ability to modulate the immune response becomes more and more evident, both in physiological and pathological processes, both in direct and indirect manners, through the biomolecules stored in granules or synthesized at the platelet level.

Regarding primary immune thrombocytopenia, this borderline autoimmune pathology with multidisciplinary implications, is generally considered benign.

The diagnosis requires the presence of peripheral thrombocytopenia in patients without comorbidities or a direct cause responsible for the thrombocytopenia.

The central ethiopathogenic mechanism of the disease is represented by the peripheral destruction of platelets by the anti-platelet antibodies that target glycoproteins on the platelet surface, the process being sustained by a dysregulation of cellular immunity at the level of T lymphocytes, with an increase in the Th1 inflammatory response and cytotoxic T lymphocytes. The persistence of autoreactivity is made possible by the reduction of peripheral immune control mechanisms, mediated by regulatory T lymphocytes. Peripheral destruction is also supported by impaired megakaryopoiesis, as megakaryocytes are similarly targeted because they express the same glycoproteins as the platelets.[1]

The central issue of this pathology is thrombocytopenia and the associated risk of bleeding.

The complexity of the disease and its management came from the fact that the diagnosis is one of exclusion, the evolution to a chronic phase is unknown, and the risk of bleeding varies among patients with the same platelet count but is difficult to be quantify accurately. Regarding treatment, it mainly relies on corticosteroids, which, for only a proportion of patients successfully controls the disease, resulting in complete responses, sustained over time. Although the initial response to corticosteroids is 60-80%, only 30-50% remain in remission [2], while over 50% of adult patients progress to the chronic phase. This underlines the fact that the pathophysiological mechanisms are multiple, being necessary to use a combination treatment that targets multiple pathogenic pathways.[3]

The absence of a biomarker regarding the diagnosis of certainty, the inability to clinically quantify the dominant pathophysiological mechanisms involved in the pathogenesis at an individual level, leads to low treatment responsiveness even in the first line of therapy, and progression to the chronic phase, which may be marked by multiple relapses and ineffective disease control, regardless of the recommended therapy.

It is known that a more targeted and aggressive therapy can result in more durable outcomes.

A deeper understanding of the disease pathophysiology and the mechanisms that leads to maintain the autoreactivity may result in new molecular therapies that aims for individualized treatment tailored to the real needs of the patient, with the goal of achieving a durable and relapse-free response. The involvement of platelets seems to be much more profound and complex than previously emphasized in the literature and appears to imply that the platelets play a pivotal role in the pathology of this disease.

Working hypothesis

Currently, immune thrombocytopenia, although is simple in definition, it is a much more complex pathology regarding diagnosis, progression, and treatment responsiveness. Even if it is considered a benign autoimmune disorder that responds to the empirically recommended treatment for all diagnosed patients that need treatment, it remains, over 250 years since its discovery, a true challenge in the clinical practice of hematologists.

At least three issues persist at the center of this pathology: the risk of bleeding and the heterogeneity of hemorrhagic syndromes among patients with the same platelet count, the risk of chronicity, and the inability to predict treatment responsiveness. These factors make the management of ITP patients extremely complicated.

The lack of a definitive biomarker for diagnosis, the inability to stratify patients into risk groups, the absence of clinical quantification of dominant pathophysiological mechanisms, the inability to accurately assess bleeding risk in practice and the need for treatment initiation, alongside the inability to correctly predict treatment response, are just a few of the unresolved issues in ITP.

The **main objectives** have focused on two major directions:

1. Utilizing data obtained from the initial evaluation to improve patient staging and predict progression to chronicity, with the aim to ensuring correct management from the first line of treatment
2. Evaluating plasmatic biomarkers and their expression across different stages of ITP evolution. Assessing proteins stored within platelets and how they contribute to

promoting immune autoreactivity, which is responsible for progression to chronic phase of ITP.

In the **General part**, the focus was on highlighting relevant aspects related to the current level of knowledge regarding the chosen topic.

Chapter 1 covers fundamental concepts related to the origin and function of platelets, detaling pathophysiological notions with an emphasis on non-hemostatic functions and how platelets participate in modulating the immune response.

Chapter 2 consideres the detailing of the theoritical notions of the disease, emphasizing the pathophysiological mechanisms and their impact on the clinical and biological profile of the patient, clinical and biological evaluation of the patient, and current treatment recommendations.

The **Personal contributions** sections begins with **Chapter 3**, which presents the working hypothesis and main objectives.

Chapter 4 includes the working hypothesis, main objectives, methodology, and results for the first research study. This study aimed to evaluate predictive factors for chronicity based on platelet count, as well as to assess bleeding risk and predictive factors for response to first-line treatment.

For this purpose, a cohort of 118 patients was selected, with a median age of 41 years, registred at the Hematology Clinic of Emergency University Hospital of Bucharest. These patients had thrombocytopenia af varying degrees and a positive diagnosis of primary immune thrombocytopenia. The median follow-up period from the time of diagnosis was 4 years. Data were used from the electronic medical records both at the onset of the disease and during subsequent follow-ups for patients with chronic forms.

The objectives of this study were:

1. Evaluation of platelet count as a predictive factor for chronicity.
2. Assessment of bleeding risk associated with the hemorrhagic syndrome.
3. Identification of other predictive factors for chronicity.
4. Analysis of clinical, biological, and immunoserological data of patients with ITP.
5. Evaluation of treatment, adverse reactions, and therapeutic response reported to the risk of chronicity, with the aim of identifying potential predictors of treatment response.

The results of this study highlight:

1. Patient distribution: the study revealed a predominant female involvement, with a female-to-male ratio of 3:1, reaching a maximum of 5:1 in the 20-40 years age group. The median age at diagnosis was 41.5 years.
2. Predictive value of platelet count: Platelet evaluation for predictive purposes can pe clinically applied, particularly from the perspective of thrombocytopenia. Analysis of thrombocytopenia severity showed that approximately 89% of patients (105 out of 118) in the study had severe thrombocytopenia (platelet count <50 x10^3/uL). Among these, 58 patients had values below 10 x10^3/uL, with a higher proportion of patients experiencing a single episode of ITP.
3. Related to the evaluation of platelet count as a predictive factor of chronicity: The statistical analysis suggested that a value of platelet count > 10 x10^3/uL correlates with a higher risk of evolution towards the chronic phase. Although it is known that a lower platelet count is associated with a higher hemorrhagic risk, the results of the statistical analysis was significant only in the comparison in pairs, having statistical significance only for the pairs 0 vs 1 (p=0.000) and 0 vs 2 (p=0.000). The hemorrhagic risk was quantified using the WHO bleeding evaluation scale, where 0 represents the absence of bleeding, 1 the presence of bleeding syndrome, and 2 the presence of mild bleeding syndrome. A positive statistical correlation was identified between the value of platelet count at diagnosis and the MPV value (p=0.034), meaning that a higher platelet count associates a higher MPV, a suggesting sign for the increased platelet turnover observed in ITP.
4. Bleeding risk evaluation: Statistical assessment of bleeding risk in ITP patients included data on the presence of bleeding syndrome, individual element from the WHO classification (type of skin manifestations, mucosal involvement, organ bleeding), presence of comorbidities, particularly infections at diagnosis, epidemiological data (age, sex), and initial paraclinical evaluation. While univariate analysis identified several statistical elements such as infections at diagnosis (p=0.026), changes in erythrocytes (p=0.002), changes in lymphocyte count (p=0.007), and thrombocytopenia <10 x10^3/uL (p=0.00), subsequent ROC curve analysis placed these variables below the reference range. Hemorrhagic manifestations, expressed by the WHO score, were more prominently represented in patients with a single episode of ITP. In other words, patients with a single episode of ITP had a platelet count < 10 x10^3/uL and associated with a more pronounce

bleeding syndrome. The risk of progression to chronicity increases in patients with an initial platelet count > 10 x10^3/uL, in the absence of bleeding syndrome.

5. For statistical analysis of predictive factors for chronicity, data from the initial patient evaluation form were analyzed, selecting only variables with statistical significance from individual tests. Continuous variables such as age, leukocytes, MPV, CRP, ESR, LDH, RBC, hemoglobin, platelet count, and nominal variables such as antiplatelet antibodies, sex, WHO score, and dyslipidemia were subjected to further statistical analysis. For better statistical representation, the WHO score was divided into 0-1 and > 2 categories.

An interesting finding from the individual variable analysis is that specific elements from the WHO bleeding scale showed significant statistical p-values: presence of gingival bleeding (p=0.007), purpura (p=0.004), ecchymosis (p=0.001), and epistaxis (p=0.007). Age, sex, presence of infections, comorbidities (autoimmune diseases, diabetes, infections at diagnosis, including Helicobacter pylori), and anti-aggregant or anticoagulat therapy were not statistically associated with predicting chronicity in ITP patients.

An intriguing aspect in the presence of dyslipidemia, which was statistically significant in univariate analysis but required confirmation in larger patients cohorts. Unexpectedly, the inflammatory syndrome was not associated with progression to chronicity.

The results of the statistical analysis are presented in the table below.

Table: Risk factors for chronicity in adult patients with ITP

Variable		Univariat Analysis (95%CI)			Multivariate analysis (95%CI)		Odds ratio
	p	Min	Max	*p*	Min	Max	
Age	0.219	0.327	0.54				
Leucocite	0.077	0.493	0.698				
MPV	0.269	0.444	0.702				
CRP	0.731	0.385	0.668				
VSH	0.885	0.339	0.686				
Erytrocyte	**0.035**	**0.508**	**0.757**	**0.022**	**1.14**	**6.591**	**2.741**
Hgb	**0.033**	**0.513**	**0.718**	**0.041**	**1.027**	**4.672**	**2.19**

Platelets	**0.00**	**0.619**	**0.815**	**0.000**	**2.464**	**12.201**	**5.483**
Pregnancy	0.037	1.027	12.504				
Sex	0.184	0.236	1.326		0.236	1.326	0.56
WHO score>2	**0.00**			**0.026**	**0.106**	**0.899**	**0.309**
Dislipidemya	0.031	0.469	0.654				
Anti platelet antibody	0.907	0.19	6.492		0.19	6.492	1.111
LDH	0.429	0.137	1.343				

The statistical validation of the obtained data was conducted using ROC curves, which established the relative risks and confidence intervals for each individual variable.

Table: Predictive Score for Chronicity in ITP

Factors	Relative risk	Points
RBC > 4.5 mil/uL	2.741	+1
HGB >13.5 g/dl	2.190	+1
PLT >10 X10^3/uL	5.483	+2
WHO bleeding scale >=2	0.309	-1

The results of the statistical analysis were used to create a predictive score for chronicity, as detailed in the table.

Respecting a uniform distribution of calculation, each parameter was asigned points based on its relative risk values, as specified in the table. The total score is the sum of points corresponding to the criteria met.

Patients are categorized into two risk groups at diagnosis, based on their total score:

- Low risk: total <2 points
- High-risk: total 2-4 points

The study was validated using the patients in the study. Calculations of risk and 95% confidence intervals (CI) confirms the statistical validity of the proposed predictive score. Patients with a high-risk score (2-4 points) have a 4.768 times greater risk of progressing to the chronic phase, with a 95% CI between 2.092 and 10.867, and a statistically significant p value (p=0.00)

Table 4.26 Statistic validation of the proposed score

		Newly diagnosed	Chronic	
Risk group	Low-risk	39	11	50
	High-risk	29	39	68
Total		68	50	118

According to the risk group classification, out of 118 patients, 68 are categorized as high risk for chronicity, while 50 are classified as low risk.

At the time of database collection, 62 patients had progressed to the chronic phase (including 12 patients who were already in the chronic phase at diagnosis). This result is close to the expected number of 68 patients with a high risk of chronicity. The difference of six patients could be attributed to the fact that some newly diagnosed patients in 2024 experienced relapses within the first three months of diagnosis, with a possibility of progressing to persistent and chronic phase over time.

A particularly interesting aspect from the statistical analysis is the presence of pregnancy. From a statistical point of view, the presence of pregnancy was identified as a predictive factor for chronicity, with a p value of 0.037, a relative risk of 3.583, and 95% CI 1.027 to 12.504. However, being a very small group of patients, this factor was considered not significant enough to be included in the predictive score for chronicity proposed in the this chapter.

Regarding first-line treatment particularities, 106 patients in our study received first-line treatment consisting of corticosteroids.

There were no statistically significant differences in clinical and paraclinical presentation, infections rates, comorbidities, treatment options, response times, and duration of response between patients who received different types of first-line treatment.

The complete response rate (platelets above 100 x10^3/uL) was 67% across the entire cohort. Out of these 106 patients, 52 experienced a relapse later. The median time to response was seven days for patients with newly diagnosed ITP and nine days for those with chronic ITP (p=0.076, not statistically significant). The median time to relapse was 6.26 months.

Variables such as age, sex, comorbidities, platelet count and MPV at diagnosis, EHO bleeding score, presence of viral markers (AgHbs, anti-HCV, anti-HIV antibodies), anemia, CRP levels, and LDH were included in the statistical analysis to identify predictors of treatment response. The result was significant only for platelet count (p=0.014).

Chapter 6 includes the second study of the thesis and how it seeks to respond to the formulated working hypothesis. The study aims to evaluate the proteomics of patients with ITP in different phases of the disease (newly diagnosed vs. chronic), with the main goal to evaluate, as a whole, the changes at the levels of some proteins belonging to classes of cytokines, chemokines, growth factors, soluble receptors, hormones, and other proteins, and how they interfere in configuring the immune response.

Secondly, the expression of proteins derived from platelets and their involvement in the immune profile of these patient categories was also monitored.

The primary objective of the study was to identify proteins with the potential to serve as plasma biomarkers useful in clinically quantifying the pathophysiological mechanisms in the evolutionary stages of ITP, followed by their individual quantification in specific patient categories to confirm their utility in clinical practice.

To achieve this, 30 patients diagnosed with immune thrombocytopenia, who were under monitoring at the Hematology Clinic of the Emergency University Hospital of Bucharest, from March 2024 and June 2024, were selected. The study groups were divided according to the current stage of the disease: newly diagnosed ITP (pool 1), chronic ITP without treatment in the last five years, some never having received treatment during evolution (pool 2), chronic ITP undergoing treatment, most receiving chronic treatment with thrombopoietin agonistic (pool 3), and the control population (control pool).

For the test, venous blood was collected from each patient, and the samples were subsequently processed to obtain platelet-depleted plasma. The analysis was performed on plasma pools, with results specific to the disease group.

The most significant changes identified by this study are found in the following classes of molecules (as shown in the figure):

- Proinflammatory cytokines: TNF-α
- Anti-inflammatory cytokines: GDF-15
- Chemokines: CXCL-5, CXCL-11, CXCL-12; CCL-11, CCL-17
- Soluble receptors: CD14, CD30, IL-1R4
- Growth factors: EGF, FGF, G-CSF, CM-CSF, M-CSF,TGF-α
- Hormones: GH, leptin, relaxin
- Proteins involves in inflammation and immune response: Crypto-1, FasL, Flt-3 ligand

While the involvement of some of these has already been highlighted in various specialized studies, for other, their clinical utility of involvement in the pathogenesis of ITP is not yet known.

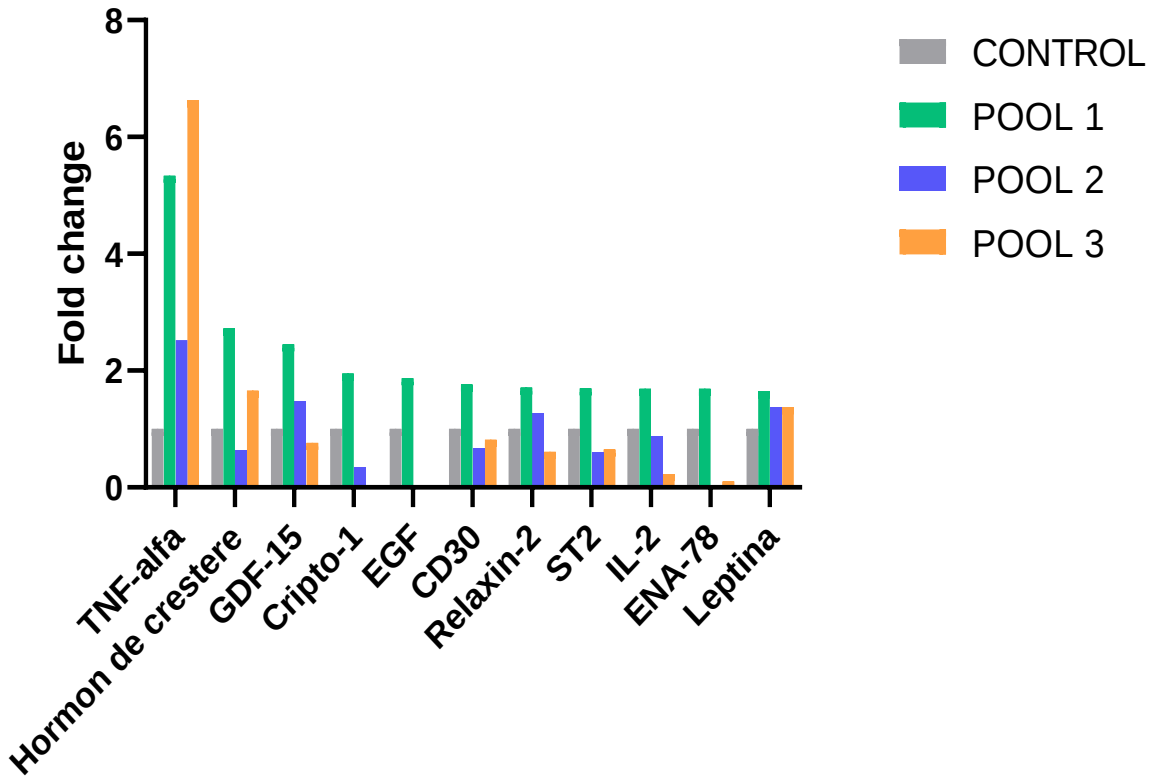

Figure 5.4 – Distribution of analyte expression in patients with different phases of ITP

Proteins with increased expression levels (at least 1.5 FC) in plasma samples obtained from the patient with acute phase immune thrombocytopenia (pool 1) compared to the healthy subjects, and how their expression changes in the other patient categories (pool 2 and pool 3).

The results obtained underline several important aspects.

Regarding the expression of proinflammatory cytokines, the present study identified significantly increased levels of TNF alpha (TNF-α) in all three patient categories. This finding is in accordance with the data from the specialized literature and supports the predominance of a pro-inflammatory type response in the pathogenesis of ITP, promoted at the level of Th1 lymphocytes.[4]

The fact that elevated value is maintained in the category of chronic ITP patients, including those undergoing treatment – some of whom have undergone multiple lines of treatment throughout the course of the disease – emphasizes that the chronicity of ITP in

these patients is most likely due to the persistence of autoreactivity at a certain, yet unclear, level throughout the progression of the disease.

Regarding **IL-2**, it shows an increased expression in the newly diagnosed ITP patient category. In patients with chronic ITP undergoing treatment, IL-2 falls below the reference value, which could mean the reversal, at least partially, of the pro-inflammatory effect with treatment. However, given that most patients included in the study were on TPO-RA treatment, a correlation between the decrease in IL-2 could be related to the treatment. The category of chronic ITP patients without treatment shows normal IL-2 levels.

Another interesting aspect is that **IFN-γ** levels are normal across all three patient categories. A possible explanation for the normal values of both IFN-γ and **IL-4** could be the small number of patients included in the study and the heterogeneity of the small number of patient groups. However, a bolder explanation might suggest that the main pathogenic mechanism in our group of patients does not involve Th1 lymphocytes.

Given that TNF-α is primarily produced by activated macrophages, while IFN-γ is produced by activated lymphocytes, we might suggest that the persistence of autoimmunity could be linked to dysfunction at the level of antigen-presenting cells rather than Th1 lymphocytes.

Recent studies have identified the **IL-17/ Il-23** axis as a key element in inflammation, playing a role in the immune response against fungal and bacterial infections, as well as in autoimmune diseases. [5]

In the present study, the changes presented in the IL-17/ IL-23 axis are minor in all categories of patients. IL-17A has values at the lower limit of normal in the chronic patient group, while IL-23 showed subnormal expression in chronic patient category. This is in contradiction with other studies that highlighted the increase in the levels of these cytokines, demonstrating their incrimination in the pathogenesis of ITP.

A possible explanation could be given by the fact that the change in the Th1/ Th2 balance observed in ITP patients varies according to the stage of the disease. In the acute phase, the increase of the pro-inflammatory response, of the Th1 type, which synthesizes cytokines like IFN-gamma and TNF-alpha, predominates. These cytokines promote the activation of macrophages and cytotoxic T cells (CTL), leading to platelet destruction, in parallel with the decrease of the Th2 response. However, in the natural evolution of the disease, there may be a change in the persistent phase toward a mixed increase in the two types of response. While the pro-inflammatory response promoted by Th1 remains active, there is also an increase in the Th2 type response, represented by the level of IL-4 and IL-

10, may become more prominent. IL-4 can inhibit the Th1-type response while promoting the differentiation of autoreactive B cell clones and the production of antiplatelet antibodies.[4]

IL-22 is primarily linked to the secretory activity of Th22 lymphocytes, but can also be synthesized by NK cells, exhibiting both pro- and anti-inflammatory properties. IL-22 showed low expression in both groups of patients with chronic ITP and normal expression in newly diagnosed ITP patients.[6]

Most studies performed on cytokines from the IL-12 family reported increased expression for all evaluated subtypes (IL-12, IL-23, IL-27, and IL-35) in chronic ITP patients, contrary to the present study, which reports low levels of IL-23 and IL-27 in chronic ITP patients undergoing treatment.[6] However, IL-12 and IL-17A levels remained normal in our patients.

The balance between IL-18 and its inhibitor, IL-18-binding protein (IL-18BP), is involved in the progression of ITP, with increased IL-18 levels and decreased IL-18BP. These correlate positively with the persistence of Th1 response in ITP. IL-18 activity can naturally be inhibited by IL-18BP, which has a high affinity for IL-18.[7]

In the current study, only IL-18BPa expression levels were measured, and contrary to expectation, they were normal across all three patient categories. Similarly, IL-12 levels were also normal in the examined patient groups. This suggests that, at least in the patients analyses, the Il-18 – IL-12 axis does not play a significant role in promoting a specific type of immune response.

IL-1β is a pro-inflammatory cytokine, produced by lymphocytes, macrophages, and monocytes during the antimicrobial response. During the immune response, PRP and TLR become expressed, leading to an increase in IL-1β expression. IL-1β stimulates CD4+ lymphocytes and their differentiation into Th17 cells. The stimulatory effect of IL-1 can be inhibited or suppressed by IL-1ra. IL-1ra is synthesized by neutrophils, macrophages, monocytes, and hepatocytes to reduce inflammation.[8]

The present study highlighted an expression of IL-1β that is far below the reference limit, especially in the newly diagnosed ITP patients and in chronic patients without treatment.

IL-33 shows low values only in the chronic ITP patients without treatment, while IL-1ra is below the reference limit only in the category of patients with chronic ITP, without treatment. Similar to our study, Zhang et al. reported low levels of IL-1β and IL-33 cytokines.[9]

The main deposit of IL-1β is found at the platelets level.[8] Following the important platelet destruction during the acute phase of ITP, the plasma levels decrease, and with this, the function in which is involved. Similarly, in the chronic phase, in patients without treatment, the reduced number of platelets may be associated with lower IL-1β level. Regarding IL-33 expression, a possible explanation can be given by the fact that IL-33 is associated with the Th2-type response, which is generally low in ITP patients.

An interesting finding from the current study is the markedly increased expression of IL-34 in the chronic patient categories.

IL-34 (stem cell factor) belongs to class III of tyrosine kinase receptor ligands, alongside macrophage colony-stimulating factor (CSF1/M-CSF), kit ligand, and FLT3 ligand (FLT3LG). These cytokines are involved in the activation of the signaling pathway through binding to the M-CSF receptor (M-CSFR). [10]They intervene in myelopoiesis, having a role in proliferation, differentiation, and functionality of polymorphonuclear cells (monocytes, macrophages). M-CSF is produced by monocytes, macrophages, dendritic cells, endothelial cells, fibroblasts, and activated T and B lymphocytes. IL-34 has similar roles to M-CSF in promoting the growth, survival, and differentiation of monocytes into macrophages. Regarding the association with autoimmune diseases, increased levels have been reported in rheumatoid arthritis, systemic lupus erythematous, and Sjogren's syndrome.[10]

Contrary to the expression of IL-34, M-CSF and FLT3L show reduced expression in both chronic ITP patient groups. Studies that have investigated M-CSF levels in patients with ITP have reported increases. The discordant results in the current study could be based on the oversaturation of M-CSFR by IL-34, ultimately resulting in an overstimulation of macrophage differentiation, most likely with a role in promoting autoimmunity by maintaining the pro-inflammatory response.

The evaluation of the **anti-inflammatory response** in this study was outlined by assessing the expression of Th2-specific interleukins: IL-4, IL-5, and Il-6, which showed normal values in all three categories of patients. IL-10 and IL-13 were also evaluated, which showed, contrary to expectations, a decrease in expression in both untreated and treated chronic ITP patients.

GDF-15 belongs to a member of the TGF-beta cytokine family and is found in all tissues. It is synthesized by macrophages in response to inflammatory stimuli such as TNF-alpha and IL-1beta, leading to the inhibition of TNF-alpha production by the macrophages. In this way, it intervenes in the immunosuppression of the inflammatory processes. Other

studies have linked GDF-15 to the inhibition of function in cells such as neutrophils, macrophages, dendritic cells, NK cells, and T lymphocytes. [11]Recently, GDF-15 has emerged as a biomarker in various chronic conditions such as cardiovascular diseases, mitochondrial disorders, diabetes, cognitive decline, aging, and in cancer research, where it seems to have a dual role – both anti-tumorigenic and pro-metastatic.[12][11,13]

GDF-15 also plays a role in anti-infectious responses. It impacts NK cell function during systemic infections and regulates macrophages polarization in adipose tissue. Overproduction of GDF-15 increases the severity of human rhinovirus infections and is associated with progression to sepsis. COVID-19 patients have elevated GDF-15 levels, suggesting a correlation with diseases severity.[13]

The direct implications of increased GDF-15 in the pathogenesis of ITP are not well known but it might represent a mechanism to counterbalance the decrease in TGF-beta resulting from platelet destruction, being known that TGF-beta is present in elevated amounts in the platelets.

At the level of chemokine class, the most important change was observed in the expression of CXCL5 (**ENA-78**), which had an increased expression in patients with active phase disease and decreased levels in both chronic patient categories. It supposed to be associated with neutrophil chemotaxis and Th2 differentiation, trough CXCL5- CXCR2.[10] It appears to have anti-imflammatory contribution in early stage of ITP.

Differences between acute ITP patients and the two chronic ITP categories were expressed for the chemokines CXCL11, CXCL12, CCL7, and CCL17. These chemokines had a normal expression in the active disease group and reduced expression in the two chronic ITP patient groups.

Among the soluble receptors, notable changes were observed for CD30 and ST2.

Soluble CD30 showed increased expression in newly diagnosed ITP patients and normal levels in both chronic ITP patient categories, while CD26 expression was normal in all analyzed categories.

Few studies have analyzed the link between CD30 and ITP, but it appears that sCD30 and sCD26 are associated with autoimmune diseases, being correlated with the Th1, respectively Th2 immune responses.[14]

Recently discovered, the IL-33/ ST2 axis is involved in Th2-type cellular-mediated immune response. The investigation of the function of the ST2 receptor has demonstrated its role in inflammatory processes, contributing to the promotion of Th2-type responses and

Th2-type cytokines. Also, it had a role of specific cellular marker involved in the differentiation of Th2 from Th1.[15]

It has been observed that the removal of the ST2 receptor favors autoimmune diseases mediated by Th1/Th17 cells. Additionally, the deletion of the ST2/ Il-33 axis favors the increase of cytotoxicity mediated by NK cells and increases the production of IFN-gamma, IL-17, and TNF-alpha. The study concludes that the presence of ST2/ IL-33 axis plays a role in attenuating pro-inflammatory mechanisms.[15]

In the current study, ST2 expression is increased in the patients with active disease, newly diagnosed, but does not correlate with IL-33 expression, which is low in patients with chronic ITP undergoing treatment, and could be interpreted as an anti-inflammatory mechanism, counterbalancing the Th1-type response.

The involvement of the IL-33/ST2 axis in ITP is not fully elucidated at the moment, but it certainly represents a subject of interest in light of these results.

1. Regarding hormone expression, increases in the expression of growth hormone (GH), leptin, and relaxin were observed.

The increased **GH** expression in patients with active disease and those with chronic ITP undergoing treatment could be correlated with the pro-inflammatory immune properties by stimulating B and T cell syntesis, with modulation of Th1/Th2.[16]

Additionally, Yang Xu et al [17], highlights in his study the ability to promote differentiation at the megakaryopoiesis level through a complementary and synergistic effect of the c-Mpl ligand expressed by megakaryocytes.

The connection between leptin and ITP has been much more intensively analyzed over time. Studies in the specialized literature have suggested that it plays and important role in the disease's pathogenesis, with increased leptin levels being associated with a decrease in platelet count. It has also been suggested that leptin acts as an anti-inflammatory agent by promoting the secretion of IL-10 from monocytes, making it a good diagnostic biomarker. [18–20]

The explanation for the increased values of **relaxin** was emphasized by D. Bani et al[21],who demonstrated in a study on mice that relaxin reduces the number of circulating platelets and decreases platelet release from megakaryocytes.

2. Other proteins involved in inflammation and immune response.

In the last group of analytes, the variation in the expression levels of other proteins involved in inflammation and the immune response was followed. Among these, a variation in patients with ITP was represented by Crypto1.

Crypto1 expression showed increased values in patients with active disease and decreased values in both categories of chronic patients.

Crypto1 is an oncoprotein of interest that belongs to the class of EGF growth factors. It functions as an extracellular signaling molecule, playing an essential role in embryogenesis, being highly expressed in tumors, where it promotes tumorigenesis. [22]

Crypto1 acts as a co-receptor for TGF-β, reducing the association between TGF-β and its receptor, TβR1. In this way, it suppresses the signaling activity of TGF-β at the cellular level, diminishing its cytostatic effects on epithelial cells.[23]

The involvement of Crypto1 in the pathogenesis of ITP patients is not known at the present but could be the subject of future studies, given the increasing emphasis in the specialized literature on its association with tumor maintenance and progression.

3. The evaluation of the involvement of platelets in the pathogenesis of ITP began with the study by Feng et al[24], which highlights the fact that CD40L, CXCL5, CCL5, and EGF are predominantly derived from platelet granules, thus suggesting the involvement of platelets in the immune response and inflammation, as well as a possible explanation for the decrease in all growth factors in the two categories of patients with chronic ITP.

A possible explanation for the increased expression of CXCL5 in acute the phases of the disease, followed by the decrease in the chronic phase, could be represented by the degree of platelet activation during immune destruction.

The exact mechanism of platelet activation in the ITP phases is unknown but an increased turnover in the acute phase could involve an increased platelet activation. The reduction or limitation of platelet clearance in chronic phases could be an explanation for the decrease in platelet-derived proteins in this study.

A central role of the proteins with increased expression in this study, whose involvement in the pathogenesis of ITP is not known, seems to converge, somehow, towards TGB-β.

A topic of interst would remain between the Crypto1 - TGF-β1 (platelt) association in the natural evolution of the disease.

At the same time, a special place in the current study was occupied by the expression of GDF-15, a member of the cytokine family of TGF-β transformation factors and its involvement in the pathogenesis of ITP. A possible role of it, suggested by the proteomic

mechanisms, especilly in the acute phases of ITP, when, along with the decrease in platelets, TGF-β also decreases greatly.

Chapter 6 brings together the conclusions and personal contributions resulting from the two studies, proving that a more thorough evaluation of the patient from the time of diagnosis, followed by risk group stratification, could improve therapeutic management and prognosis.

Study 2 highlights the persistence of a pro-inflammatory response in chronic phases, even more so in categories of patients requiring chronic therapy, both through known mechanisms and through pathways that have not yet been researched, emphasizing at the same time the fact that the platelet seems to be more than just a target molecule, more changes in protein expression in the study converging towards its immune role.

Therefore, extensive, individualized studies are still necessary in order to identify biomarkers accessible in clinical practice that will guide us in the correct management of ITP patients, especially for that cases of multiple refractory patients with an increased risk of bleeding.

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