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Biological and clinical picture in Immune Thrombocytopenia (ITP) patients: prospective cross-sectional data on a single centre population

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Abstract

Immune thrombocytopenia (ITP) is an autoimmune disease characterized by isolated thrombocytopenia (PLT < 100.000/mmc). Patients may be asymptomatic at presentation, or they may present with mild mucocutaneous to life threatening bleeding. Irrespectively of bleeding problems, patient with ITP often report fatigue and health-related impaired quality of life. The incidence of ITP ranges from 2 to 4 cases per 100.000 person-years and although some patients experience only one episode of thrombocytopenia followed by immediate remission, more than 70% of patient present persistent or chronic ITP.

The pathophysiology of ITP is complex and remains incompletely understood. Traditionally anti-PLT autoantibodies are considered to have a key role in PLT premature destruction, but nowadays an increasing relevant role is quoted on breakdown in self-tolerance which drives to autoimmune cell-mediated and autoantibodies mediated processes, causing PLT destruction with different mechanisms, including inadequate PLT production by megakaryocytes impaired by T-cell and autoantibodies and abnormalities of T-cell, such as skewing of T helper (Th) cells towards type 1 helper (Th1) and type 17 helper phenotype and a reduction in numbers and function of regulatory T cells (Treg), which could enhance autoimmune process.

Diagnosis remains a process of ruling out other causes of thrombocytopenia, as there is no diagnostic test for ITP; antiplatelet antibodies is detected only in 50% of ITP patients, with low specificity and sensitivity, therefore this test is note recommended in the diagnostic workup, which includes collection of medical and drug history, physical examination, blood smear, blood tests on biochemistry,

viral and serology screen, haemolysis screen, haematinics, blood proteins and electrophoresis.

Treatment is recommended in bleeding patients and below 30.000/mmc PLT count. Glucocorticoids (dexamethasone and prednisone) are recommended as first line treatment. In case of bleeding and/or PLT count < 10.000/mmc, IVIG is indicated in association with steroids, but unfortunately only 30 to 50% of adults have a sustained response after glucocorticoids are discontinued. Those patients who present persistent or chronic ITP, in order to guarantee a safe PLT count (>30.000/mmc) and to prevent bleeding, undergo second and subsequent line of treatment, with thrombopoietin-receptor agonists (TPO-RA), such as eltrombopag, romiplostim and avatrombopag, immunosuppressive agents such as Rituximab, azathioprine, mycophenolate mofetil, ciclosporin, fostamatinib (an oral spleen tyrosine kinase (Syk) inhibitor), or splenectomy which remains highly effective options inducing long-lasting remissions in 60 to 70% of patients. In the absence of biomarkers to guide the choice of medication, treatment is selected on other factors, including adverse effects, required speed of response, drug-to-drug interaction, patient and clinician preference, drug availability.

Although not yet validated, role of biomarkers has been investigated for diagnosis, monitoring and treatment response prediction.

This single centre prospective cross-sectional study aims to collect both clinical and biological information on ITP patient population in charge among Haematology department Genoa, San Martino Hospital.

Biological data collection includes testing for cytokines, biochemical and immune markers among the ITP patient population.

For each enrolled patient clinical data were collected and reported, including state of disease at time of assessment, type, number and timing of previous and/or ongoing treatments, patient quality of life perception, disease history.

Data collected provided a biological picture at one-point time of a heterogeneous ITP patient population at different timepoints of their disease course, connecting information with clinical data (state of disease, type of previous or ongoing treatment and response, Quality of Life data).

The main goal of this project was to collect a biological snapshot of ITP patients from this single centre population, providing synchronous clinical information in order to both compare results with the available published data and have a starting point for future longitudinal studies.

Abbreviations

Below the table of acronyms and abbreviations:

ITP	Immune Thrombocytopenia
PLT	Platelet
QoL	Quality of life
HRQoL	Health Related Quality of life
ICH	Intracranial haemorrhage
CR	Complete response
PR	Partial Remission
MHC	Major Histocompatibility Complex
APC	Antigen presenting cell
TCR	T-cell receptor
DCs	Dendritic Cells
pDCs	plasmacytoid Dendritic Cells
Tregs	regulatory T cells
Bregs	regulatory B cells
CTL	Cytotoxyc T Lymphocytes
IFN	Interferon
TGF	Transforming Growing Factor
TNF	Tumor Necrosis Factor
IL-	Interleukin
MIF	Macrophage Inhibition Factor
MDSC	Myeloid-derived suppressor cells
APA	Antipaltelet Antibodies
TPO	Thrombopoietin
c-Mpl	Myeloproliferative Leukemia Protein
TRO-RA	Thrombopoietin receptor agonist
CMBs	Cerebral Microbleeds
MRI	Magnetic Resonance Imaging
ADCC	Antibody-Dependent Cell-mediated Cytotoxicity
AMR	Ashwell-Morell receptors
FcγR	IgG FC-portion Receptor
CRP	C-reactive protein
FcRn	Neonate Receptor Fc portion
MCH-I	Major Histocompatibility Complex class I
TRIM-21	Tripartite Motif-containing protein 21
FNAIT	Fetal or Neonatal Allo-Immune Thrombocytopenia
HDFN	Haemolytic Disease of the Fetus and the Newborn
PNH	Nocturnal Haemoglobinuria and
aHUS	Atypical Heamolytic Ureamic Syndrome
CAPS	Catastrophic Antiphospholipid Syndrome
CAR-T	Chimeric Antigen Receptor T-cell therapy
BAFF	B-activating protein
YAP	Yes-associated protein 1

1. Background on Immune Thrombocytopenia biological and clinical features

1.1 ITP Definition and epidemiology

Immune thrombocytopenia is an acquired autoimmune disease characterized by isolated thrombocytopenia (PLT < 100.000/mmc) resulting from platelet destruction and impaired platelet production [1,2].

As isolated thrombocytopenia is a common finding, potentially due to many different conditions, diagnosis is process of ruling out other causes of decreased platelet count, both immune-mediated (secondary ITP), or non-immune. Differently from primary ITP, secondary ITP is a broad term that includes all forms of immune-mediated thrombocytopenia that are due to an underlying autoimmune diseases (such as systemic lupus erythematosus, antiphospholipid syndrome), immunodeficiency states (such as IgA deficiency and common variable immunodeficiency), lymphoproliferative disorders (such as B Chronic Lymphocytic Leukaemia, Large Granular Lymphocytic Leukaemia, lymphoma) or infections (such as HIV, Helicobacter Pylori, CMV, hepatitis B, hepatitis C); secondary immune thrombocytopenia can also develop following drug exposure (heparin, quinidine) [3].

Patients with immune thrombocytopenia may be asymptomatic at presentation or they may present with mild mucocutaneous to life threatening bleeding.

Although only 5% of patients present with severe bleeding, bleeding leading to hospital admission develops in approximately 15% of ITP patients within 5 years after diagnosis [2].

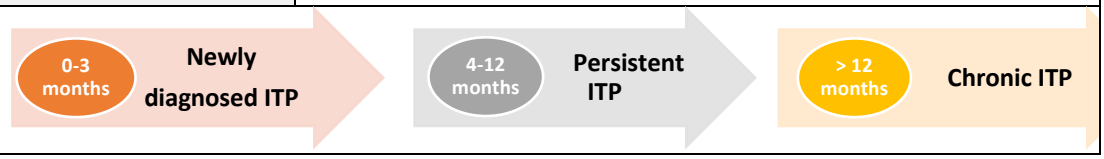
Intracranial haemorrhage (ICH) has been reported in 1.4% of adults and 0.1-0.4% of children with ITP. Adults with ITP have a 1.3- to 2.2-fold higher mortality compared with the general population, due to cardiovascular disease, infection and bleeding. [2] Irrespective of bleeding problems, ITP patients often report fatigue and health-related impaired quality of life (QoL) [1,2].

The incidence of ITP ranges from 2 to 4 cases per 100.000 person-years, with two peaks: one between 20-30 years of age with slight female predominance and one after 60 years of age with equal sex distribution [2].

Although some patients experience only one episode of thrombocytopenia followed by immediate remission, more than 70% of adult patient develop persistent (since 3 to 12 months after diagnosis) or chronic (more than 12 months after diagnosis). The likelihood of a spontaneous remission from ITP is therefore age related, being unlikely in adults and high in young children (1-year remission rates 74% in children 1 year of age, 67% in children 1 to 6-year-old, 62% in those 10 to 20 years of age, 20-40% in adults) [1,3,4].

Definition, standard terminology and phases of disease (**Table 1**) have been defined for ITP by an International Working Group of expert clinicians [4].

Table 1. ITP phases of disease (adapted from [4])

ITP Phases of disease [GL6]	
Newly diagnosed ITP	within 3 months from diagnosis
Persistent ITP	between 4 to 12 months from diagnosis. Includes patients not reaching spontaneous remission or not maintaining complete response off therapy.
Chronic ITP	Lasting for more than 12 months
	

1.2 ITP Pathophysiology

The pathophysiology of ITP is complex and remains incompletely understood. Traditionally anti-PLT autoantibodies are considered to have a key role in PLT premature destruction, as antibody coated PLT are destroyed in spleen and liver, and autoantibodies may induce complement-induced or desyalilation-induced PLT destruction through Fc-mediated phagocytosis in reticuloendothelial tissues. However, presence of anti-PLT autoantibodies is irregular and other mechanisms described are T-cell dysregulation and inadequate PLT production by megakaryocytes impaired by immune system. Autoimmune process may be driven by abnormalities of T-cell, including skewing of T helper (Th) cells towards type 1 helper (Th1) and type 17 helper (Th17) phenotype and a reduction in numbers and function of regulatory T cells (Treg); CD8+ T lymphocytes might be

involved too. Cytotoxic T cells may also have a direct lytic effect on circulating platelets or bone marrow megakaryocytes [2,5].

In the following small paragraphs, the main pathophysiology mechanisms will be briefly reported.

PLT production

Megakaryocytes are giant precursors cells that grow in the bone marrow and go throw endomitosis, becoming polyploid, developing abundant cytoplasm rich in cytoskeletal proteins and complex membrane system. Driven by microtubule-based forces and cytoskeleton, megakaryocytes' cytoplasm develops long extensions called proplatelets, storing on their tips ∝-granules, mitochondria, and other granules. Pre-platelets are released from the tips of proplatelets into the blood stream where they undergo further fission into platelets, 2-3 micron anucleate disks of megakaryocyte-derived cytoplasm, living 7-10 days, and undergoing eventually destruction mainly in the liver, as described below.

All these steps of platelets formation, maturation ageing and destruction are potential target of the immune attack on platelets or their precursors, with different mechanisms which are described in the following paragraphs [5].

Thrombopoietin

Thrombopoietin (TPO) in a hematopoietic cytokine produced by the liver, represents the primary regulator of megakaryocyte progenitor expansion and differentiation. Moreover, it is well known that TPO plays a key role for the

maintenance of haematopoietic stem cells, acting as a pan-haematopoietic cytokine.

Through its specific receptor, c-Mpl (Myeloproliferative Leukemia Proteine), expressed on haematopoietic stem cells and on platelets, TPO activates multiple and complex signalling pathways, promoting cellular survival and proliferation.

Noteworthily, cMpl receptor provides a mechanism for the regulation of circulating TPO level: if the PLT count is high more c-MPL receptors are available to bind and internalise TPO, reducing the TPO level and consequently both megakaryocytes and platelets formation. Conversely in thrombocytopenia less TPO in internalised by circulating platelets which are reduces, and more TPO remains available in circulations and binds megakaryocytes stimulating thrombopoiesis. Interestingly, in ITP patients TPO levels are not particularly raised compared with non-immune thrombocytopenia, and this may be due to the rapid PLT turnover rate. A second negative feedback mechanism has been described for TPO circulating levels related to TPO mRNA expression in hepatocytes directly affected by hepatic internalisation of platelets related to their level of attached glycoproteins (see below paragraph on “Platelets desialylation”) [6,7].

The knowledge developed above this molecule, which was cloned more than 30 years ago, allowed the development of TPO receptor agonists (TPO-RA) drugs and represents an example and an opportunity of treatment targeting [8].

Interestingly, there is data available showing an inverse relationship between endogenous TPO level and response to treatment TPO-RA in patients with ITP, patients with lower or normal endogenous TPO levels ($\leqslant$100 pg/ml) showed

increased rate and depth of response to TPO-RA, while patients with significant TPO elevations (>200 pg/ml) were unlikely to have a satisfactory response to those agents [9].

Platelet desialylation

Glycoprotein GPIbα, a membrane-bound receptor largely expressed on platelets' surface, has an extracellular domain with an abundance of N-linked glycans. When the terminal sialic acid groups attached to glycoprotein are lost or reduces, senescent platelets are recognised by hepatocytes (by Ashwell-Morell receptors, AMR) leading to internalisation and intracellular signalling activation (JAK2-STAT3 pathway) which drives to increased hepatic TPO mRNA expression, resulting in raising TPO circulating level, megakaryopoiesis stimulation and platelets mass restoration. The above describes mechanism show the relationship between desialylation and platelet lifespan. It has been described an increase of Platelet desialylation in ITP patients, with a correlation with presence of autoantibodies, not only anti-GPIb but also anti-GPIIb/IIIa. Interestingly, Oseltamivir, an antiviral agent inhibiting viral neuraminidase used to treat Influenza A and B, showed correlation to platelets count increase when administered to ITP patients, likely due to its action on reducing activity of human sialidases/neuraminidase [7].

Platelet apoptosis

Physiologically, platelet lifespan, which is approximately 10 days, is regulated by apoptotic processes activated in aged platelets. Apoptotic pathway in platelets is

regulated by the critical balance between pro-survival proteins, such as BCL-X_L and their role in restraining the pro-death activity of the “multidomain killers” BAD, BAK1 and BAX. With platelets ageing, the balance gradually moves in favour of the latter, driving to process of mitochondrial membrane permeabilization, cytochrome C release, caspase activation and finally apoptosis. As in the process of desialylation-mediated platelet removal, the liver is the site of clearance of apoptotic platelets. ITP patients appear to have an imbalanced expression of pro- and anti-apoptotic and increase of caspase and alteration of mitochondrial membrane has been described [7].

Platelet immune role and interaction with CD8+ T cells

PLT and megakaryocytes are known to present antigen to T cells and activate naïve cells to induce immune response. Moreover, PLT release extracellular vesicles that load antigens in Major Histocompatibility Complex class-I (MHC-I) molecules and promotes CD8+ cells proliferation.

PLT interactions with leukocytes are known to play a role in the pathobiology of immune, inflammatory and thrombotic responses and this has been particularly studied in sepsis, where immune suppression and thrombocytopenia are common. Data published about clinical and experimental sepsis, show significant upregulation of PLT-leukocytes interaction and marked increase of PLT-associated MHC-I, as PLT internalize and process exogenously loaded antigen, generating peptides that are loaded onto MHC-I.

Increased PLT MHC-I during sepsis was also shown to be associated with enhanced antigen cross-presentation by PLT as well as enhances antigen-

specific interaction with CD8+ T cells. This upregulation of PLT-leukocytes interaction and increase of PLT MHC-I studied in the sepsis model, may have implications on pathophysiology of immune and inflammatory disease, including ITP [5].

Anti-PLT autoantibodies

Host factors have a known role in development and persistence of ITP, as the immune system recognizes platelet proteins as foreign antigens. Traditionally a key role has been recognized in autoantibody production and consequent peripheral thrombocytopenia. Famous Harringhton-Hollingsworth experiment showed that plasma from patients from ITP caused significant cytopenia when reinfused in healthy volunteers [10]. Some years later, Shulmat et al. identified the “ITP factor” as an antibody, promoting ITP t be considered as a prototype for Ab-mediated autoimmune disease [11].

However, only a half of ITP population have detectable antiplatelet autoantibodies and other immune mechanism have been studied (see below paragraphs) [12].

According to most recent ITP guidelines, antiplatelet antibodies (APA) testing is considered an adjunct not mandatory laboratory test owing to its low sensitivity and specificity and consequent limited role in diagnostic flowcharts for ITP [1,13]. Moreover, there are various APA laboratory testing techniques available, such as glycoprotein-specific testing and monoclonal antibody immobilization of platelet antigens (MAIPA) assay, and poor standardization due to irregular testing implementation in clinical practice. Nevertheless, potential clinical utility

of this testing beyond diagnosis has been suggested, such as correlations between certain serologic patterns and response to treatment [14].

Consistent laboratory data collections in ITP patients are available and different serologic patterns have been described, such as antibodies against glycoproteins GPIIb/IIIa, GPIb/IX and GPIa/IIa, with different implication on a prognostic land treatment response level [15–17].

More than 50% of ITP patients present autoantibodies against glycoproteins, majority of them IgG dominant, being IgG1 the most common subclass, either alone or together with other subclasses; only a small minority of patients present IgG2, IgG3 or IgG4 alone. Remarkably, in many of the autoantibodies analysis studies published across decades evidence of oligoclonality was reported for a proportion of patients, while others showed polyclonal antibodies production [18–20].

IgG Fcγ-receptors (FcγR)

Immunoglobulins exert their biological effects by several effector systems. IgG affects innate and adaptive immune systems throughout complement activation (see below) and binding their FC-portion with the Fcγ-receptors. These are a family of cell surface receptor expressed on leukocytes: FcγRIa, FcγRIB, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa and FcγRIIIb. A part from FcγRIIb, which exhibits an inhibiting function, all the above receptors, expressed on monocytes, activated neutrophils and macrophages, activated by binding of IgG FC-portion, stimulate immune response by inducing phagocytosis of the IgG-opsonised target,

Antibodies-Dependent Cell-mediated Cytotoxicity (ADCC), stimulating release of inflammatory mediator and superoxide radicals [21].

According with these mechanisms, IgG-opsonised platelets undergo FcγR-mediated phagocytosis, via recognition of IgG-FC portion by macrophages receptors (FcγR), particularly FcγRIII, eventually resulting in thrombocytopenia. Degree of FcγR response depends on multiple factors, such as antibody titre, IgG isotype affinity to Fcγ- Receptors (ie. IgG2 only recognise FcγRIIa, while IgG1, IgG3 and IgG4 all receptors, at slightly different affinity), genetic polymorphisms, infections (molecular mimicry, antibody cross-reactivity), inhibitory immune receptors signal regulatory proteins [22].

Additionally, a role for C-Reactive Protein (CRP) in enhancing PLT-opsonised phagocytosis has been described. Remarkably, acute phase protein CRP, produced in liver and upregulated during inflammation and infection, in response to IL-6 and IL-1, once binding on IgG-opsonised platelets' surface to ligand phosphoryl choline in a calcium-dependent manner, is able to potentiate the uptake and degradation of phagocytes [23].

Moreover, two other FC receptors are expressed ubiquitously in almost all cell the neonatal Fc-receptor (FcRn), a homologue of the Major Histocompatibility Complex class I (MCH-I) molecule, and the Tripartite Motif-containing protein 21 (TRIM-21), a ubiquitin-protein ligase, both receptors are expressed inside cells. FcRN is expressed on vacuoles and tubules, binding and transporting intracellularly both IgG and its cargo, together with albumin in a pH-dependent manner, recycling both ligands and therefore extending their half-life. FcRN has additional functions, other than intracellular transport and recycling of IgG, such

as transport across cellular barriers (endothelial, epithelial, syncytiotrophoblast), resulting in mucosal and mother-to-child IgG transport, and participation in IgG-mediated phagocytosis in myeloid cells, delivering immune complex-bound antigens into the antigen presenting pathway, boosting secondary responses. On the other side, TRIM-21 is highly represented in cytosol and binds IgG-opsonized target to ubiquitination and proteasome degradation, stimulating via intracellular signalling cascades (ie. Scr- and Syk-kinases) and transcription factor pathway such as NF-kB, secretion of pro-inflammatory cytokines, modulation of natural killer stress ligands and cell degranulation, resulting in initiation of cellular responses, such as phagocytosis (by monocytes, macrophages and neutrophils) or ACDD (by NK cells and myeloid cells). These processes are utilized beneficially by cells to eliminate invalid pathogens such as viruses or cancer cells, but have been described to be detrimentally involved in auto- or allo-immune conditions, for instance in Ab-mediated platelets destruction in ITP or FNAIT (Fetal or Neonatal Allo-Immune Thrombocytopenia) or in Ab-mediated red cells destruction in HDFN (Haemolytic Disease of the Fetus and the Newborn) [21].

Immunoglobulin glycosylation patterns

IgG antibodies are glycoproteins containing a highly-conservative sugar component attached to an asparagine (ASN297) in the antibody Fc domain; this glycan is essential for their functional structure and is required for binding IgG with FcγR. Normally this glycan consists of a core of N-acetylglucosamine and mannoses with variable levels of galactose, sialic acid, N-acetylglucosamine and

fucose. Variation of this glycan compositions affects antibodies affinity to FcγR with consequences on Ab effector activity. According with in vivo and in vitro evidence, the enrichment of sialic acid (sialylation) considerably reduces Ab affinity to FcγR, being this effect particularly relevant in the protective role of IVIG in IgG-binding. On the other hand, core fucosylation has been demonstrated to be highly relevant in Ab function as a lack of core fucose results in a marked increase of binding affinity restricted to FcγRIIIa/b. Interestingly, a decrease of total IgG1 Fc fucosylation has been described in ITP patients, with related increase of PLT phagocytosis and antibody-dependant cellular toxicity (ADCC). Similarly, a trend of increased IgG1 Fc-galactosylation has been observed in ITP patients with increased ADCC and affinity to FcγR binding [21,24,25].

Complement activation

The complement system provides a link between adaptive and innate immune system. Autoantibodies, particularly IgG, may fix complement activating the classical complement system. Many disorders due to complement dysregulation present with thrombocytopenia, such as Paroxysmal Nocturnal Haemoglobinuria (PNH), Atypical Heamolytic Ureamic Syndrome (aHUS) and Catastrophic Antiphospholipid Syndrome (CAPS), but in these conditions the pathophysiology is related to defects in alternative pathway and terminal complement regulation. In these disorders, thrombocytopenia is not usually severe and the clinical most relevant aspects are haemolysis and thrombotic complications due to endothelial damage.

Noteworthily, platelets show on their surface many proteins active in complement pathways such as binding sites for C1q, receptors for complement cleavage proteins, C3a and C5a, capable of inducing platelet activation and thrombo-inflammation, complement regulatory proteins, CD55 and CD59. Platelets are protected from the activation of platelet-bound C3 by the alternative pathway inhibitor of complement activation, platelet-derived factor H, therefore C3 activation on PLT is primarily mediated by the antibody-dependent classical pathway [26].

Plasma from ITP patients have showed to be able to fix complement to platelets in vitro. IgG autoantibodies, mainly IgG1, present in ITP are potent activators of the classical complement pathway, particularly when assembled in hexamers [27]. Moreover, it has been recently suggested a role for Fc galactosylation in enhancing C1q binding and subsequent complement activation [26,28].

ITP patients have been studied for plasma level of complement C3, C4 and CH50 which are usually reported to be in normal range when compared to other immune complex-related disorders. However, some recent studies comparing ITP patients complement level to healthy control show lower mean C4 and CH50 serum levels, without any predictive role of response to treatment. However, low C4 levels did predict severe ITP [28].

The role on antibodies-activated complement, potentially contributing to PLT destruction in ITP patients, is under investigation, opening potential therapeutic targeting, which is under investigation [12,29].

Soluble Factors

Many abnormalities have been demonstrated in ITP patients' sera in regards to soluble immune mediators such as chemokines and cytokines. As early as in the seventies, an increase of MIF (Macrophage inhibition factor), a T-cell derived factor, was described in ITP patients [30]. Following this first finding, many alterations in chemokines/cytokines have been described, the most remarkable being the high level of Th1 and Th17 cytokines known to be related with active ITP manifestation and most likely due to a lack of Tregs suppression of autoimmunity. As PLT contain high level of Tumor Growth Factor- β, TGF-β, able to induce Tregs production, increase of PLT count in ITP patients after treatment is able to reverse the above relationship between high level of TH1/Th17, lower Tregs activity ad ITP disease manifestations [12,31,32].

Recently the current knowledge in cytokines profile in ITP and its potential implication for diagnosis, treatment and prognosis has been reviewed by Andreescu *et al*., appearing to be an heterogeneous profile, with different patterns observed in different subset of patients. Specifically, there is evidence of imbalance of pro-inflammatory cytokines (Interleukin-6, IL-6, and Tumor Necrosis Factor-α, TNF-α) and anti-inflammatory cytokines (IL-10, TGF-β), secreted mainly by Treg cells. Some ITP patients have been described to express Th1-type cytokines profile characterized by elevated level of TNF-α and Interferon-γ, IFN- γ, and IL-2, while others to present a Th2-type cytokines profile with high level of IL-4, IL-5, IL-6, IL-10 and IL-13, with evidence of a possible switch between the two patterns during disease course, being Th1 immune response upregulated in acute phase, with rebalancing and even Th2

polarization in subsequent phased of the disease. Accordingly, all cytokines related with Th1 activity (TNF-α, IFN-γ, IL-2) are usually increased in acute phases, but also an increase of Th2 related cytokines (IL-4, IL-5, IL-6, IL-10 and IL-13) have been described throughout disease history. Additionally, IL-18, a cytokine that promotes Th1 responses, is increased in ITP patients and the balance between IL-18 and its receptors (IL-18BP) has been described as variable alongside disease progression [28].

According with above findings, many studies aimed to investigate cytokines'' level in ITP patients and possible correlation with diagnostic, prognostic and therapeutic aspects of disease. Interestingly, the level of anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β have been described to be increasing during and after steroid treatment. With another elegant study by Stimpson and colleagues, not only the relationship between steroid treatment and cytokines level and trends was investigated further, but it was also connected with steroid treatment response. In fact, a relative abrogation of IL-10 and persistence of IL-17 was demonstrated in patients who failed steroid treatment, representing potential biomarkers for steroids refractory disease [28,33].

Defects in antigen presenting cells

Recognition by T helper of their antigen in association with MHC on Antigen Presenting Cells (APC) is essential in developing IgG responses, including autoimmune autoantibodies production. Different cell can act as APC: Dendritic Cells (DCs), macrophages, under particular circumstances B cells and even platelets and megakaryocytes can play this role.

Many reports have demonstrated DCs impairment in ITP. Elevated expression of CD86 demonstrated on DC surface in ITP patients may be related with stimulation of autoreactive T cells. Low number of plasmacytoid DCs (pDCs) was described in ITP and significantly correlated with low PLT count as lack of type I interferon secreted by pDCs is supposed to play a role modulating activated autoreactive T cells in ITP. Lastly, marked reduction of DC-associated indoleamine 2,3-dioxygenase-I was shown in ITP patient, which is supposed to impair the differentiation of regulatory T cells (Tregs), contributing to the observed Tregs deficiency in ITP.

In addition to the known and relevant DCs impairment, macrophages appear to play a key role in ITP pathophysiology. Not only macrophages are the primarily phagocytes responsible for splenic destruction of autoantibodies-opsonised platelets, but they also process and present PLT autoantigens to autoreactive T helper cells, feeding a continuous autoantigen-autoreactive-autoimmune loop which needs to be broken by immunosuppressive therapy. The increased antigen presenting capability in ITP macrophages may be enhanced, particularly when inflammation or infection occurs, by the upregulation of MHC-II molecules on macrophages surface stimulated by inflammatory cytokines such as IFN-γ [12].

T cell lymphocyte dysregulation

T cell lymphocytes undergo thymic maturation, with positive selection and maturation if bearing TCRs with low or intermediate affinity, while those with high affinity undergo negative selection and apoptosis, in order to prevent the recognition of self-antigens.

T-cell immune response applies by recognition of antigens on MHC molecules on the surface of APC. Furthermore, T-cell precursors differentiate in effector T-cell and memory T-cell, namely cytotoxic T lymphocytes CTL CD8+ and T helper cells Th1 CD4+ and Th2 CD4+. The effector response of Th1 and Th2 involves the secretion of various cytokines (see above paragraph) with Th1 mainly associated with inflammation and Th2 with allergic response and stimulation of B cell proliferation. T regulatory cells (Tregs) maintain immune homeostasis by suppressing autoreactivity in the peripheral blood, directly inhibiting the function of CTL and Th cells by direct cell interaction and anti-inflammatory cytokine release such as IL-10, TGF-β [28].

Half of ITP patient have detectable autoantibodies against platelets glycoproteins, but a significant role of T-cell in the immune mechanism involved in ITP has been demonstrated both in presence and absence of humoral effectors. Specifically, in ITP individuals there is evidence of platelets destruction by cytotoxic T lymphocytes (CTL), which, stimulated by APC and T helper cells, exert their cytotoxic function by inducing apoptosis and contributing to PLT lysis. Additionally, the balance between Th1 and Th2 results to be altered, while a dysregulation and breakdown of self-tolerance with Tregs dysfunction and decrease are described. The above T-cell-mediated mechanisms are variously connected with the B-cell dysregulation and presence of autoantibodies.

Specifically, Th1/Th2 balance, which is crucial for normal immunity, with Th1 mainly associated with inflammation and Th2 with allergic response and stimulation of B cell proliferation, is often assessed by cytokines levels as the distinction between Th1 and Th2 subset in based mainly on IFN-γ and IL-4

production, respectively. Studies have demonstrated that the Th1/Th2 ratio in substantially higher in ITP patients compared with healthy controls, indicating a potential role for Th1/Th2 polarization in the pathogenesis of ITP. Conversely, different studies suggest Th2 polarization, suggesting a likely variability of the Th1/Th2 balance during different phases of disease, with Th1 immune response upregulation in acute phase, with rebalancing and even Th2 polarization in persistent and chronic stages [28].

As a result of many threads of research, nowadays it is well established that ITP is, at least partially, due to a lack of peripheral T cell tolerance mechanism, related to defective Tregs activity. Reduced CD8+ T cell suppression and defective and reduced number of CD4+ T regulatory cells is likely responsible for excessive production of anti-platelets autoantibodies. There is evidence of rescue of Tregs deficiency in ITP following PLT count increase following TPO therapy [12].

Interestingly, together with a Th1 stimulation and increase of pro-inflammatory cytokines, CTL CD8+ toxic activity against platelets and megakaryocytes raises, as PLT membrane possess the membrane proteins (ie. MHC-I) and the intracellular potential signalling and structure (ie. caspases, mitochondria) to represent a target for CD8+ monitoring and destruction via activation of apoptosis [34].

Increasing interest on T-cell role in ITP pathophysiology moved research grout to study further T-cell compart in different phases of the disease. Malik and colleagues managed to demonstrate the presence of T-cell CD8+ clones in ITP

patients with active disease by mean of characterization of TEMRA (Terminal Differentiated Effector Memory)[35].

NK cell lymphocyte dysregulation

Several observations showed that in ITP patients NK cells numbers in the peripheral blood may be normal or reduced, particularly in children, but NK cytotoxic activity results to be suppressed and, as NK cells are known inhibitors of B cell differentiation and affinity maturation, this impairment of NK cytotoxicity may potentially be influencing autoantibodies production in ITP patients [5].

B-cell dysregulation

Although the role of B-cells in ITP pathophysiology, namely in producing autoantibodies, is well established, therapeutic approach targeting B-cell such as anti-CD20 monoclonal antibodies therapies (ie. Rituximab) in not as satisfying as we could have expected, with a considerable percentage of patient failing to respond and another relevant group of the relapsing early after treatment.

Explanation for this may reside in the heterogenous diverse mechanism behind immune dysregulation in ITP. Recently some new elements have been described regarding transcriptional factors involved in megakaryocyte maturation and function: YAP (Yes-associated protein 1), together with well-known GATA1 protein, is supposed to have a role in maintaining appropriate cytoskeletal function and consequently MK adequate maturation and platelets production. Reduced level of YAP and GATA1 have been described in ITP patients with potential future therapeutic target implications [36].

On a different level, possible explanation for persistent B-cell dysregulation regardless target treatment could be the demonstrated permanence of plasma cells and memory cells in spleen, sometimes with proved clonality. Long-Life Plasma Cells (LLPC) have been largely demonstrated in spleen of ITP patients, also after Rituximab treatment, although only in bone marrow the plasma cells should find the favourable environmental to prolong indefinitely their lifespan, as their longevity depends on microenvironment and is favoured by niche of other haematopoietic cells. Noteworthily, only recently the role of BAFF (B-activating factor) a pro-survival cytokine of TNF family and its relative APRIL, have been described, showing that an increased level of these cytokines, produced mainly by neutrophils and immune cells, may be responsible in ITP patients for LLPC in spleen [37]. These findings offered a potential new therapeutic target in BAFF/APRIL molecules, and clinical trials testing the efficacy and safety of the combination Rituximab and Belimumab (a specific inhibitor of BAFF) are ongoing [38].

Moreover, in light of the potential role of plasma cells and memory cell in spleen and bone marrow, B-target approaches such as anti-CD 38 Daratumomab and anti-CD18 CAR-T (Chimeric Antigen Receptor-T) therapies are under investigation [39,40].

Bregs and other myeloid and lymphoid cells involved

B regulatory cells (Bregs) are reduced in number and defective in function in ITP patients, consequently production of anti-inflammatory cytokine IL-10 is reduced and monocytes TNF-α inhibited.

Bregs are potent immunoregulatory cells and they appear to be reduced peripherally in ITP patients and potentially increased in spleen. Similarly to Tregs, Bregs number and function increase after TPO therapy [12].

Myeloid-derived suppressor cells (MDSC), population of myeloid progenitor cells acting as potent regulator of adaptive immunity, having the ability to inhibit T cells proliferation by starving the cells by nutrients required, have been showed to be abnormal and reduced in number in patients with ITP. Corticosteroids treatment demonstrated showed activity in rescuing MDSC number and restore Tregs function [12,41,42].

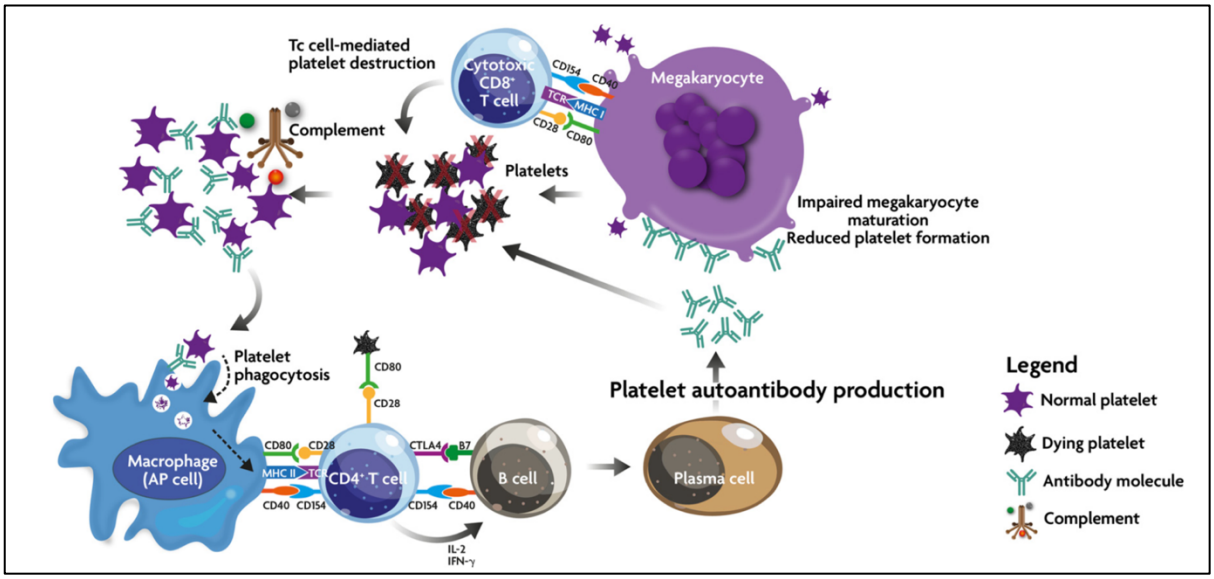
ITP pathophysiology: summary

Although presence and clinical relevance of autoantibodies has been largely demonstrated in immune thrombocytopenia, as only a half of ITP population have detectable antiplatelet autoantibodies, other immune mechanisms have been hypothesized, including reduced megakaryocyte platelets production/secretion, defects of antigen-presenting cells, presence of cytokines/chemokines, complement protein or other soluble immune mediators, and immune cells dysregulation. The new evolving module of the immune and autoimmune responses in ITP is a complex picture of stimulation and suppression of lymphocytic function, platelet production and platelet destruction, where B and T cells defects are central in ITP pathophysiology, as it appears that PLT autoimmunity is caused by a failure in immune tolerance (self-tolerance breakdown) [12]. Moreover, activated platelet may play a role in immune

stimulation, even in triggering and propagating their own destruction, particularly by antigen presentation to CD8+ cells [5].

Provan *et al.* provided a graphical representation of the main pathophysiology characteristics, reported in **Figure 1**.

Figure 1. ITP Pathophysiology [12]



Extracted from Provan *et al.* [12]: "Immune effector mechanism in ITP. Due to a breakdown in self-tolerance, APC (including megakaryocytes) process and present platelet autoantigens to autoreactive T cells, which then begin a cascade of events including stimulation of autoantibody production and cytotoxic T cell activation. These two mechanisms lead to peripheral platelet destruction and megakaryocyte inhibition in the bone marrow. In addition, autoantibody-opsonized platelets may come under the attack of the complement cascade".

1.3 ITP Clinical features

ITP main clinical manifestation is low PLT level. Patients may present this laboratory finding and being completely asymptomatic or show symptoms and related morbidity.

Bleeding patterns

Patients with immune thrombocytopenia may be asymptomatic at presentation or they may present with mild mucocutaneous to life threatening bleeding. Although only 5% of patients present with severe bleeding, bleeding leading to hospital admission develops in approximately 15% of ITP patients within 5 years after diagnosis. [2] Patient with a PLT count > 30.000/mmc rarely develop spontaneous bleeding, however there is no correlation between degree of thrombocytopenia and the occurrence or severity of bleeding [43].

Intracranial haemorrhage (ICH) has been reported in 1.4% of adults and 0.1-0.4% of children with ITP. Although clear ICH is pretty rare in ITP patients, observational data showed Cerebral microbleeds (CMBs) to be present in more than 40% of ITP patients who had experienced at least one nadir < 30.000/mmc and studied with MR imaging. CMBs or microhaemorrhages are tiny hemosiderin deposits, which can be detected in the brain noninvasively using susceptibility-sensitive magnetic resonance imaging (MRI) techniques. Differently to other older populations, where the presence of CMBs is associated with cognitive impairment in specific disease conditions, in ITP patients, who effectively develop dementia more often and at a younger age compared to other populations, no relationship between dementia and MCBs have been demonstrated so far [44].

Mortality

Adults with ITP have a 1.3- to 2.2-fold higher mortality compared with the general population, due to cardiovascular disease, infection and bleeding. Incidence of

venous thromboembolism (VTE) is twice as high among ITP patients as among general population [1].

Fatigue, Quality of Life and other symptoms

Irrespectively of bleeding problems, ITP patients often report fatigue and health-related impaired quality of life (QoL) [1,2].

Large data collection reports perception from patient's and doctors' points of view. Majority of patients reported that ITP reduced their energy levels (85%), capacity to exercise (77%), and limited their ability to perform daily tasks (75%). Additionally, ITP-related fatigue comes together with marked reduction of HRQoL perception (80%), reduction of emotional well-being (49%) and increase of worries in regards to future (63%). In the same survey it was reported that, because of ITP, patients find themselves decreasing in work productivity, and consequently willing of reducing working hours, and considering terminating their employment. Noteworthily, the subgroup of younger adult and adolescence showed peculiar pattern of needs and emotional and psychological impact by the disease [45–47].

COVID-19 and ITP

During COVID-19 outbreak clinical practice in ITP patient care was strongly impacted. According with the known potential relationship between infection and triggering of ITP, an increase in incidence of ITP cases has been related to SARS-CoV2 infection. PLT count, disease features, bleeding and complications risk are often peculiar in COVID-19 positive patients and treatment strategies,

according with published data and recommendations, should consider the unique clinical context and the concomitant viral infections with its potential complications. Accordingly, a warning on limiting the use immunosuppression in ITP management and favouring other treatment approaches was followed all around the world, where applicable and safe [48].

1.4 ITP Diagnosis

Diagnosis remains a process of ruling out other causes of thrombocytopenia, as there is no diagnostic test for ITP; antiplatelet antibodies is detected only in 50% of ITP patients, with low specificity and sensitivity, therefore this test is note recommended in the diagnostic workup. ITP diagnostic workup includes collection of medical and drug history, physical examination, blood smear, blood tests on biochemistry, viral and serology screen, haemolysis screen, haematinics, blood proteins and electrophoresis. Other additional tests can be considered. Bone marrow is not diagnostic in ITP, therefore it is not mandatory at the initial workup and can be performed only in those patients in which other haematology abnormalities is suspected or in which response to first line treatment was inadequate [1,2,13].

Misdiagnosis is common and, particularly when response to treatment is not satisfying alternative diagnosis should be considered, including hereditary thrombocytopenia, particularly if family history is consistent [2].

Below a table reporting main mandatory and optional workup tests according with published ITP recommendations [1,13]. Main mandatory and optional workup tests were reassumed in **Table 2**.

Table 2. Main mandatory and optional workup tests [1,13]

Mandatory ITP work up test	**Tests to be considered in ITP workup**
Medical History	Pregnancy test (if applicable)
Physical examination	Thyroid function
Full blood count	DAT, Direct Antiglobulin Test
Peripheral blood film	Fecal Ag Helicobcater Pylori
Biochemistry	Viral PCR for EBV, CMV and parvovirus
Plasma proteins and quantitative Ig level	Autoimmunity tests
Haematinics (ferritin, vitamin B12, folate)	Antiphospholipid Autoantibodies
Haemolysis screen (haptoglobin, LDH, bilirubin, reticulocytes)	Imaging (i.e. abdomen ultrasound, chest X-ray)
Clotting screen, including fibrinogen	Additional bleeding test
HIV, HCV, HBV serology	Blood group
Citrate test (EDTA-dependent platelets aggregation)	Bone marrow aspirate and trephine (in selected patient)

1.5 ITP Treatment

Goals of treatment are generally aimed to stop bleeding, to reduce risk of bleeding recurrence and to improve QoL in ITP patients [43].

Treatment is recommended in bleeding patients and for PLT count below 30.000/mmc PLT count. Glucocorticoids (dexamethasone and prednisone) are recommended as first line treatment. In case of bleeding and/or PLT count < 10.000/mmc, IVIG is indicated in association with steroids. In life threatening situation PLT transfusion and antifibrinolytic agents should be considered to stop bleeding. Although 60 to 80% of patients with ITP have an initial response to

glucocorticoids, only 30 to 50% of adults have a sustained response after glucocorticoids are discontinued. Those patients who present persistent or chronic ITP, in order to guarantee a safe PLT count (>30.000/mmc) and to prevent bleeding, undergo second and subsequent line of treatment [1,2,12].

Second line treatment available are thrombopoietin-receptor agonists (TPO-RA), such as eltrombopag, romiplostim and avatrombopag, immunosuppressive agents such as Rituximab, azathioprine, mycophenolate mofetil, ciclosporin, fostamatinib (an oral spleen tyrosin kinase (Syk) inhibitor), or splenectomy which remains highly effective options inducing long-lasting remissions in 60 to 70% of patients [1,12,49].

According with emerging pathophysiology theories, new treatment are in clinical development, such as anti-FcRn, Bruton tyrosine kinase inhibitors (BTKi), drugs targeting complement (C_{IS} inhibitors, factor B inhibitors), new TPO receptor agonists, proteasome inhibitors, anti-CD38, BAFF/APRIL inhibitors [12].

In the absence of biomarkers to guide the choice of medication, treatment is selected on other factors, including adverse effects, required speed of response, drug-to-drug interaction, patient and clinician preference, drug availability.

1.6 Role of biomarkers in clinical ITP management

Although not yet validated, role of biomarkers has been investigated for diagnosis, monitoring and treatment response prediction (**Table 3**).

Table 3. Biomarkers and their potential role in TP management [33,50–52]

Biomarker	Possible diagnostic and/or prognostic role
IL-1, IL-18, IL-36, IL-33	Ability to distinguish between primary and secondary ITP
IL-10 e IL-17	Markers of steroid refractory ITP
IL-2, IL-11, IFN	Markers of Th1 type T helper cytokine response
GPIV, GPIb/IX, GPIIb/IIIa autoantibodies	Expression of autoimmune disease
miRNA (99, 182-5p, 183-5p, 130°, 409-3p expression, lncRNA TMEV PG1	Epigenetic control of cell-cell adhesion, ubiquitin mediated proteasome degradation, mRNA non - sense mediated decay
PB lymphocyte immunophenotyping	T-cell and B-call asset
Expression of TRAIL in megakaryocyte	Megakaryocyte maturation index
CXCL4, CXCL13	Maturation index, effect on immune response
FC gamma receptors (FCGR) IIb expression in macrophages	Correlation with HP infection
Gut microbiome	Effects on immune response
Complement C3 and C4 fractions	Complement activation caused by GP IIb/IIIa antibodies
IPF, immature PLT fraction	Distinguish between consumption thrombocytopaenia and ITP
TRAF6 level	Response to glucocorticoids
PLT desyalylation	Response to glucocorticoids
Telomere length and telomerase activity	Correlation with outcome and bleeding
PLT and megakaryocyte microparticles	Procoagulant characteristics
Lipoprotein	Markers of oxidative stress
Haptoglobin	Marker of response to splenectomy

2. Project outline, results and discussion

2.1 Aims of the study

1. To collect biological data, such as biochemical, soluble and immune markers expression, on ITP patient population, aiming to provide data at different timepoints of ITP disease course.
2. To collect clinical data on the above ITP patient population, including state of disease at time of assessment, type, number and timing of previous and/or ongoing treatments, patient quality of life perception, timeline of disease history.
3. To observe biological patterns and their possible correlation with clinical picture, response to treatment lines and disease course.
4. To compare the above data with the available published data on biochemical, soluble and immune markers expression and their possible role on disease course prediction, including treatment response.
5. To design a future longitudinal study on clinical and biological course of ITP disease at different timepoints on the centre population.

2.2 Materials and methods

Study design

This is a prospective cross-sectional single-center study including patients diagnosed with immune thrombocytopenia (ITP) aged ≥16 years followed at the

Haematology outpatient department of San Martino Hospital in Genoa (Italy) between 01st March and 30th September 2024.

Medical and research team

Two different medical teams were involved in the study and patients' enrolment: Haematology unit, IRCCS San Martino Hospital Genoa (Hospital unit) and Haematology clinic, IRCCS San Martino Hospital and University of Genoa (University unit).

Patient enrolment

ITP patients from the centre were screened and if eligible to be enrolled in the cross-sectional study an informed consent was presented for signature. All adult ITP patients in follow up in haematology clinic in IRCCS San Martino, Genoa, were considered for enrolment.

Inclusion criteria:

- clinical diagnosis of ITP
- age > 16 years
- signed informed consent
- full mental capacity to fill up quality of life questionnaire
- available reliable clinical data on patient disease history.

Exclusion criteria:

- declined informed consent

- age < 16 years
- concomitant haematology malignancy
- clinical or safety reason against blood sample collection.

Assessment point

According with the cross-sectional structure of the study, for each patient an assessment time was scheduled. The assessment timepoint matches the moment in which biological samples were collected and clinical information on disease state refers to (**Table 4**).

Table 4. Clinical data collection

For each patient enrolled the following clinical information were collected and reported	
Diagnosis	Date of diagnosis, PLT count at diagnosis
Medical history	- Past medical history - Comorbidities (haematology disease, autoimmune disease, bleeding conditions…) - Concomitant medications
Workup (see table 2)	Full blood count with differential and Peripheral Blood smear Liver Function Test (LFT) and Renal function Serum protein electrophoresis and immunoglobulins Virus screen and serology Haemolysis screen Haematinics (vitamin B12, folate, ferritin) Bone marrow examination if indicated
Bleeding	Any bleeding episode among the whole disease history is recorded: date, severity, treatment at the time of bleeding episode, need for hospitalization, need for medical or surgical bleeding control
State at assessment point:	At the assessment point the following clinical data are recorded: - ongoing treatment or only follow up - if ongoing treatment: type of treatment, dose, date of start - PLT count - clinical situation (ECOG, objective examination, symptoms, bleeding) - concomitant medications

Previous treatment	For each treatment line received the following data are reported: - type of treatment - PLT count at the beginning of treatment - date and duration of treatment - response to treatment
Treatment categories:	1. No treatment 2. steroids (prednisone or dexamethasone) 3. TPO-RA (Eltrombopag, Romiplostim, Avatrombopag) 4. Splenectomy 5. Fostamatinib 6. Rituximab
Patient categories on the basis of steroid response:	1. *Only Follow up*: PLT count always > 30.000/mmc, never received treatment, never had bleeding episode 2. *Steroid responders*: good and stable (always > 30.000/mmc) response to a single steroid treatment course 3. *Steroid not responders:* no response to one or more steroide course and need for 2nd or subsequent line treatment 4. *Steroid dependent:* temporary response to steroids, lost with dose weaning and need for 2nd or subsequent line treatment 5. *Steroid intolerance*: steroid course not tolerated and need for 2nd or subsequent line treatment
Response criteria:[4]	- Response: PLT count > 30.000/L and at least 2-fold increase of the baseline count ad absence of bleeding - Complete Response (CR): PLT count > 100.000/L ad absence of bleeding - Non-response: PLT count < 30.000/L or less than 2-fold increase of the baseline count or bleeding
Quality of life	Quality of life at assessment point was assessed by a specific questionnaire applied to all enrolled patients

Study procedures

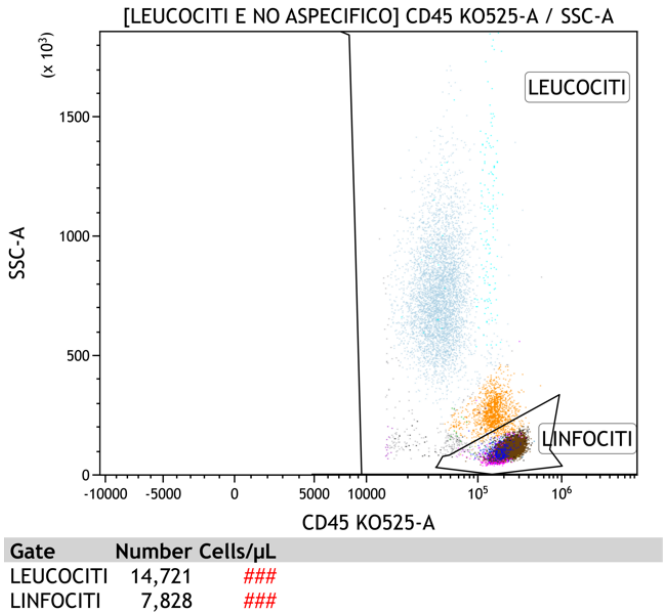
Demographic, clinical and laboratory characteristics were collected for all study participants at the assessment point. Variables explored in the study (see table 4) included: age, sex, comorbidity, time from diagnosis to study inclusion, type of ITP specific treatment (including steroids, TPO-RA, Rituximab, Immunoglobulins ev, Fostamatinib, immune suppressive drugs) and their specific dosage and administration schedule, splenectomy if performed, concomitant anticoagulant and antiplatelet therapy, concomitant pregnancy status, duration of follow-up, and biochemical variables (including white blood cell count, PLT count,

biochemistry, serum protein electrophoresis and immunoglobulins, ferritin, lipoprotein, complement markers, hemolysis markers, clotting tests and vonWillebrand functional test, immunophenotyping on peripheral blood).

Cytofluorimetric analysis was performed fresh, in specific lab, aiming to define and describe immunophenotypically leukocyte and lymphocyte population, to dercibe any abnormality and identify clonal population in present.

Below **Figure 2** is showed, extracted from a patient cytofluorimetric analysis which is extensively reported at attached 1.

Figure 2. Immunophenotype reporting



Biological data collection and storage

All patients enrolled underwent a blood sample collection for soluble, biochemical and immune markers testing. Frozen or fresh sample were processed in local clinical and research laboratory. Biological data tested on each single patient blood sample were selected on the basis of Table 3 contents.

Peripheral blood samples were collected at the same timepoint and stored in different evacuated anticoagulant-containing tubes such as 3-5 ml tubes containing Na^+ EDTA, 3–8 ml tubes containing lithium heparin (LH/PST), 3-5 ml tubes containing acid citrate dextrose (ACD). For each patient a total of 6 tubes (two for each anticoagulation system) were collected, and after centrifugation with swinging bucket rotor, frozen and stocked at -80°C fridge for delayed second level biological, soluble, immunological and cytofluorimetric testing. Biological sample were stored as: plasma (liquid part of blood that remains after the suspended cells have been centrifuged; contains clotting factors and has dissolved electrolytes), serum (similar to plasma but without the clotting factors, and buffy coat (fraction of blood that mostly contains white blood cells and platelets and white blood cells).

Some of the biochemical analysis were performed among the main hospital laboratory. Immunophenotyping analysis was performed by the local cytofluorimetry laboratory. Cytokines tests were performed for IL-4, IL-10, IL-17, IFN-γ and TGF-β by the local haematology and immunology research laboratory, with “Quantikine Elisa kit”, ® Bio-techne.

Quality of life questionnaire

Additionally, at the assessment timepoint was proposed to all patients enrolled, a 30-question multiple choice Quality of Life (QoL) questionnaire and, if accepted, filled anonymously, allowing the study team to collect directly from patients’ data on personal experience, emotional aspects, perspective on symptoms, physical and psychological aspects related to disease. Models of QoL

questionnaire available on publications were followed to design our own questionnaire [45–47].

Definitions

Immune thrombocytopenia (ITP) was defined according to International Guidelines as an autoimmune disease characterized by isolated thrombocytopenia (PLT < 100.000/mmc) [1,4]. Among the patient population, process of diagnosis was carried out by performing a full work up as recommended by guidelines and patient selected accordingly. In regards to distinction between primary and secondary, all primary ITP patients were included and patient affected by secondary ITP were considered for enrollment only if the concomitant condition was not included in study exclusion criteria and was not clinically relevant [53].

According to our study protocols the study population was divided in 6 different categories, aiming to identify different patterns of clinical course and treatment response.

1. **No treatment:** patient affected by ITP who have never undergone ITP treatment as PLT count remaining steadily and consistently above treatment threshold (> 30.000/mmc)
2. **Steroids:** patient who received one or more steroids treatment cycle and no other treatment lines.
3. **TPO-RA**: patients who are receiving TPO-RA treatment at different dose regimens, including decreasing doses schedule, aiming for

discontinuation. Patients who received different treatments before TPO-RA are included in this category, but splenectomised patients are not.

4. **Splenectomy:** all patients who underwent splenectomy along their disease history were included in this group, irrespectively of present PLT count or current or previous treatments.
5. **Fostamatinib**: patients who were receiving treatment with Fostamatinib (regardless number or type of previous treatment line).
6. **Rituximab:** patients who had received Rituximab as last ITP treatment.

Ethics

The study was carried out in accordance with the declaration of Helsinki. Informed consent was signed by all patient enrolled.

2.3 Results

The study population included 88 patients diagnosed with ITP, whose clinical and demographic characteristics were resumed in **Table 5**. Overall, the median age at time of ITP diagnosis and study enrolment were 49.0 (IQR 33.0-64.9) and 58.8 years (IQR 42.6-72.0), respectively. Most patients were female (50 out of 88, 56.8%) and mostly of Caucasian ethnicity (85 out of 88, 96.6%). Regarding comorbidities most patients reported 1 to 3 concomitant diseases (65 out of 88, 96.6%), while 18 (20.4%) and 5 (5.7%) reported no and more of 3 concomitant diseases, respectively. The main comorbidities reported were represented by cardiovascular diseases, endocrinological and rheumatological/autoimmune

diseases in 19, 18 and 12 patients respectively. Three out of the 50 female patients were pregnant. In the study population few patients (5 out of 88, 5.6%) were also receiving concomitant anticoagulant and/or antiplatelet therapy. Splenectomy was performed for treatment of ITP in 8 out of 88 patients (9%).

Patients' characteristics

Table 5. Clinical and demographic characteristics of the study population

Baseline characteristics	**N°**
Total patients included	**88**
Mean age at ITP diagnosis (IQR), y	**49.0 (33.0-64.9)**
Median age at enrolment (IQR), y	**58.8 (42.6-72.0)**
Sex	
Female	**50 (56.8)**
Male	**38 (43.2)**
Caucasian ethnicity	**85 (96.6)**
Number of comorbidities	
0	**18 (20.4)**
1-3	**65 (73.9)**
>3	**5 (5.7)**
Type of comorbidities	
Cardiovascular disease	**19**
Endocrinological disease	**18**
Rheumatologic and autoimmune disease	**12**
Gastrointestinal disease	**9**
Solid tumor	**9**
Neurological disease	**8**
Thrombotic events	**7**
Musculoskeletal disease	**5**
Diabetes mellitus	**5**
Chronic lung disease	**4**
Urologic disease	**3**
Anemia	**3**
Obesity	**2**
Chronic kidney disease	**2**
MGUS	**2**
Pregnancy	**3 (3.4)**
Concomitant anticoagulant and/or antiplatelet therapy	**5 (5.6)**
Splenectomy performed	**9 (9)**

IQR: interquartile range; MGUS: monoclonal gammopathy of undetermined significance

Every subject of the 88 patients' population was diagnosed with ITP by performing a full work up as recommended by guidelines [1,2,4]. As much as 40 patients out of 88 (45%) were assessed by bone marrow aspirate and trephine, usually before starting a second line treatment in a steroid resistant/dependent patient.

Patients' categories

Table below reports categorization of patients' population in different groups, number of patients for each group and subcategory describing clinical course (**Table 6**).

Table 6. Clinical course specifics

6 patients' groups	N° patients	Clinical course specifics
1. No treatment	21	All patients being clinically and laboratory maintaining PLT count > 30.000
2. Steroids	26	Patients maintaining after steroids PLT account as follows: - 30-50.000/mmc (2pts) - 50.100-100.000/mmc (11 pts) - > 100.000/mmc (13 pts)
3. TPO-RA	27	TPO-RA administration regimen: - Stable dose (14 pts) - Reducing dose regimen (11 pts) - Combination with steroids (2 pts) - Switch between TPO-RA drugs (8 pts)
4. Splenectomy	8	Actual management: - Off treatment, good PLT level (2) - TPO-RA at various regimens (6)
5. Fostamatinib	2	- Monotherapy (1) - Combination with steroids (1)
6. Rituximab	4	Disease status and treatment: - Remission > 12 months off treatment (3) - Early combination treatment (1)

ITP-specific treatment

In regards to ITP-specific treatment and response, 67 patients out of 88 received steroids as first line treatment and 26 achieved good and stable response after steroids cessation, while 41 patients needed second line treatment as proved to be purely “steroids not responders” (4) or “steroids dependent” (37).
Often together with steroids, in severe thrombocytopaenia and bleeding episodes, IVIG were administered in 41 patients along their disease history.

As second or following line, 37 patients of our study population received TPO-RA, 23 and 3 Eltrombopag and Romiplostim respectively, while 8 patients underwent switch and sequential therapy. TPO-RA treatment was ineffective only in 3 patients, needing combination with steroids and/or subsequent treatment lines. In 2 patients TPO-RA was discontinued for complications (recurrent VTE and allergic reaction).

In regard to Fostamatinib use, only 5 patients received this treatment as third or following line, 2 of them achieved response.

Rituximab was offered to 13 relapse/refractory ITP patients from our study population over their disease history; 5 of them proved to be primary not responders, in one patient administration was permanently suspended for allergic reaction CTCAE gr 3-4. Among Rituximab-responder patients 2 achieved a permanent response (> 18 months), 1 experienced early relapse (< 6 months) and 1 relapsed after 12 months.

As many as 6 refractory ITP patients received other immunosuppressive treatments, such as cyclosporine, cyclophosphamide, azathioprine, mycophenolate, vincristine, etc.

Laboratory results

All enrolled patients went through laboratory investigation. Below we report some results from the tests performed for the whole population and for all 6 specific groups. Laboratory altered results of the study population are reported for each different group in **Table 7.**

Table 7. Laboratory features

Lab abnormality	Total Population	Patients Categories
Leucocytosis (WBC > 10,000 /mmc)	9 patients	1. No treatment: 0 2. Steroids: 2 3. TPO-RA: 5 4. Splenectomy: 1 5. Fostamatinib: 0 6. Rituximab: 1
Low Fibrinogen (< 2.0 g/L)	3 Patients	1. No treatment: 0 2. Steroids: 1 3. TPO-RA: 1 4. Splenectomy: 1 5. Fostamatinib: 0 6. Rituximab: 0
Low Von Willebrand functional test (< 50%)	10 patients	1. No treatment: 0 2. Steroids: 2 3. TPO-RA: 8 4. Splenectomy: 0 5. Fostamatinib: 0 6. Rituximab: 0
High Reticulocytes count (> 1.7 x 100 RC)	20 patients	1. No treatment: 3 2. Steroids: 3 3. TPO-RA: 11 4. Splenectomy: 1

		5. Fostamatinib: 1 6. Rituximab: 1
Decreased Haptoglobin (< 0.3 g/L)	6 patients	1. No treatment: 3 2. Steroids: 1 3. TPO-RA: 2 4. Splenectomy: 0 5. Fostamatinib: 0 6. Rituximab: 0
Increased LDH (> 225 U/L)	17 patients	1. No treatment: 3 2. Steroids: 1 3. TPO-RA: 11 4. Splenectomy: 0 5. Fostamatinib: 1 6. Rituximab: 1
Reduced C3/C4 (< 0.9/<0.1 g/L)	13 patients	1. No treatment: 4 2. Steroids: 3 3. TPO-RA: 4 4. Splenectomy: 0 5. Fostamatinib: 1 6. Rituximab: 1
Increased lipoprotein (> 75 nmol/L)	9 patients	1. No treatment: 3 2. Steroids: 1 3. TPO-RA: 3 4. Splenectomy: 1 5. Fostamatinib: 0 6. Rituximab: 1
Reduced Ig (IgG/IgA/IgM < 8,0 <0.7 < 0.3)	23 patients	1. No treatment: 4 2. Steroids: 9 3. TPO-RA: 6 4. Splenectomy: 3 5. Fostamatinib: 1 6. Rituximab: 0
Paraprotein on serum electrophoresis	9 patients	1. No treatment: 2 2. Steroids: 3 3. TPO-RA: 3 4. Splenectomy: 0 5. Fostamatinib: 0 6. Rituximab: 1

Immunophenotyping analysis was performed on peripheral blood.

The following table (**Table 8**) reports results on the population and on the specific groups.

Table 8. Immunophenotypic features

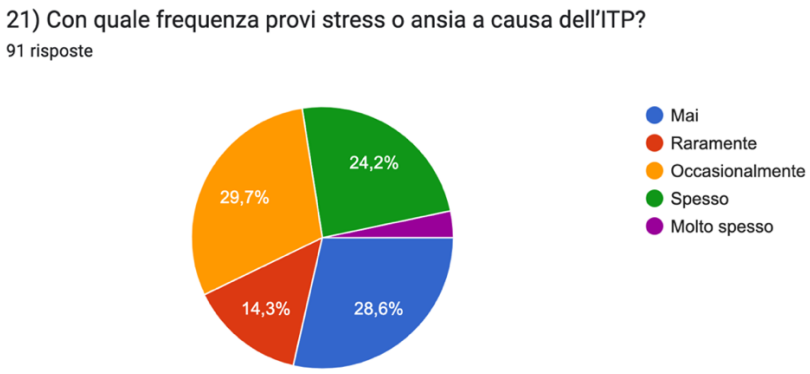
Immunophenotypic abnormality	Total population	Patients Categories
Lymphopenia B CD19+	28 patients	1. No treatment: 10 2. Steroids: 5 3. TPO-RA: 7 4. Splenectomy: 3 5. Fostamatinib: 1 6. Rituximab: 2
Lymphopenia T CD3 + CD4+	11 patients	1. No treatment: 1 2. Steroids: 4 3. TPO-RA: 4 4. Splenectomy: 1 5. Fostamatinib: 0 6. Rituximab: 1
Lymphopenia T CD3 +CD8+	9 patients	1. No treatment: 3 2. Steroids: 1 3. TPO-RA: 4 4. Splenectomy: 1 5. Fostamatinib: 0 6. Rituximab: 0
Lymphopenia NK	16 patients	1. No treatment: 3 2. Steroids: 7 3. TPO-RA: 5 4. Splenectomy: 0 5. Fostamatinib: 1 6. Rituximab: 0
Lymphocytosis B CD19+	4 patients	1. No treatment: 0 2. Steroids: 2 3. TPO-RA: 0 4. Splenectomy: 2 5. Fostamatinib: 0 6. Rituximab: 0
Lymphocytosis T CD4 +	4 patients	1. No treatment: 0 2. Steroids: 2 3. TPO-RA: 1 4. Splenectomy: 1 5. Fostamatinib: 0 6. Rituximab: 0
Lymphocytosis T CD3+CD8+	2 patients	1. No treatment: 0 2. Steroids: 2 3. TPO-RA: 0 4. Splenectomy: 0 5. Fostamatinib: 0 6. Rituximab: 0
Lymphocytosis NK	3 patients	1. No treatment: 1 2. Steroids: 1

		3. TPO-RA: 1 4. Splenectomy: 0 5. Fostamatinib: 0 6. Rituximab: 0
Deranged ratio CD4/CD8	24 patients	1. No treatment: 5 2. Steroids: 10 3. TPO-RA: 5 4. Splenectomy: 2 5. Fostamatinib: 1 6. Rituximab: 1
Clonal B lymphocytes with k/λ restriction	3 patients	1. No treatment: 0 2. Steroids: 1 3. TPO-RA: 1 4. Splenectomy: 1 5. Fostamatinib: 0 6. Rituximab: 0
Suspect T cell population	2 patients	1. No treatment: 1 2. Steroids: 1 3. TPO-RA: 0 4. Splenectomy: 0 5. Fostamatinib: 0 6. Rituximab: 0

QoL questionnaire results

Extended graphics reporting results of each single question are included in Attached 1. As many as 91 patients accepted and filled the anonymous questionnaire. Here we report the graphical answer at one of the 30 questions included in the questionnaire (**Figure 3**).

Figure 3. Patient questionnaire results example



2.4 Discussion, implication in clinical practice and future perspectives

This prospective cross-sectional study provides biological and clinical data on 88-ITP patients sample from our haematology institution.

Biological pattern described in our patient population can be declined on 6 subgroups identified according with clinical disease course, including treatment response, aiming to describe the differences between the groups and compare to the available published data.

However, there are significant limits in this study. Firstly, our patients' population is small and from a single centre (two haematology units). Limits of a small sample are particularly relevant when we analyse results for the small subgroups (some of the groups have < 5 patients) which makes it challenging to apply a statistic comparison between groups and with the available published results.

In addition, the cross-sectional structure of the study only provides a snapshot of the "here and now" biological picture of a heterogeneous ITP patient population (at different timepoints of their disease).

Moreover, due to local laboratory capacity limitations, only part of the described ITP biomarkers has been investigated up front, while some of the tests, performed on stocked samples, are ongoing (such as cytokines dosing) and some of them have been delayed to subsequent research phases in order to optimize their role and costs for a phase of the study with longitudinal structure which may potentially provide more reliable and significant results.

Nonetheless, having the above limits in mind, this cross-sectional study may be the starting point for designing and performing a prospective longitudinal study,

involving ITP patients' population from our centre, aiming to assess the variables in different phases, from clinical initial onset throughout disease history by scheduling collection of clinical and biological material at particular timepoints, such as before and after any treatment line, investigating different relationship between biological patterns and disease course and treatment response in the single patient and describing possible change of biological pattern during disease history.

Clinical data collected on our sample, compared with descriptive available data, appear to be coherent in regards to proportion of patients responding to first line steroids treatment, patients requiring a second line treatment and different treatment response rate and tolerance. Interestingly, in our study the sample size of patient in TPO-RA is large (compared to other groups) and reflects good response rate to this treatment, as expected, even in patient who are heavily pre-treated, which points out the clinical relevance, also in our clinical institution, and the specific challenges of this patients group, such as the implementation of reducing dose protocols aiming for treatment discontinuation (11 patients out of 27 on TPO-RA are on a reducing dose schedule recording positive data on safety and success in dose reduction).

Data on splenectomised patients, on bleeding incidence and on treatment tolerance and effectiveness from our patients' population reflects what is expected according to published data. Noteworthily for the small sample, but expected as per bigger size available data, among the TPO-RA group 8 patients

out of 27 successfully performed switch between TPO-RA drugs achieving good response on PLT count, without toxicity.

Biological data collected showed consistent results, although statistic comparison between groups was not considered reliable due to small sample size and heterogenous patients characteristics (such as different disease and treatment history), as discussed above. Immunophenotyping analysis showed alteration in virtually all patients, particularly T, B or NK lymphopenia or lymphocytosis, of various entity, and occasional findings of very small clonal or atypical B or T populations. Undeniably, it is markedly challenging to correlate these findings to clinical practice as they hardly have the potential to affect clinical decision, both on diagnostic and a therapeutic level. However, these findings may have a role on a research level, as matching this information with soluble and biochemical findings (i.e. cytokines level expression) may help in refining the laboratory analysis and identifying particular biological patterns which, on future longitudinal studies, might be followed up throughout disease history and timepoints, observing for possible modification with treatment.

Findings recorded up on other biological variables, such as reduced complement level, high level of lipoprotein and positive haemolysis markers in absence of clinically relevant haemolytic anaemia, are notable with again the limit of poor clinical application but a potential interest in applying these variables to a prospective longitudinal analysis.

Remarkably, vonWillebrand functional tests resulted significantly deranged in more than 10% of patients with a clear connection (> 60%) with presence of

paraprotein, as well described in the pathogenesis of Acquired vonWIllebrand conditions. It is noteworthy that this patient, even with irregular and erratic platelet count, do not present a remarkable bleeding pattern. However, in light of frailty of these patients and concomitant thrombocytopenia, including vonWillebrand screening while performing diagnostic ITP work up can be considered, particularly in patient presenting with concomitant paraprotein.

This project can also have a potential impact on clinical management of ITP patient in our centre. As a University and research hospital, it is particularly relevant in IRCCS San Martino, Genoa, to deliver high quality and evidence-based care and, at the same time, implementing systematic data collection and research practice in daily clinical practice. This project can be starting point of future continuous and systematic clinical research planning on ITP clinic.

The relevance of a Quality of Life survey is, moreover, remarkable, due to the well-known impact of diagnosis, treatment and medical burden on this particular patient population. In light of this, this project may also have a role on raising patient involvement in their own clinical care and, at the same time, on raising health professional team awareness on patient quality of life issues, on patients' perspective and on importance of patients' empowerment, aiming enhancing an integrated, value-based and patient-centred care for ITP patients.

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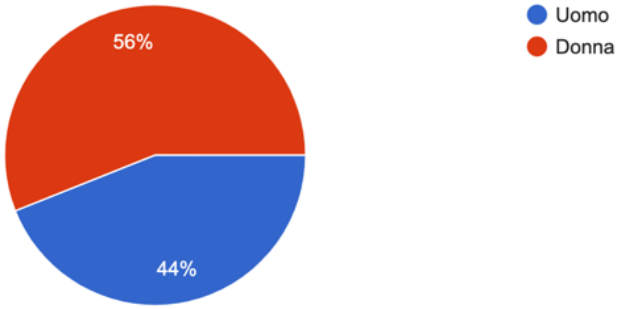
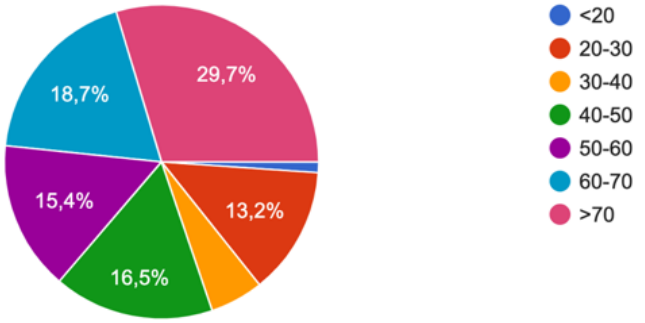
Attachments

Attachment 1. Main graphics on QoL questionnaire answers

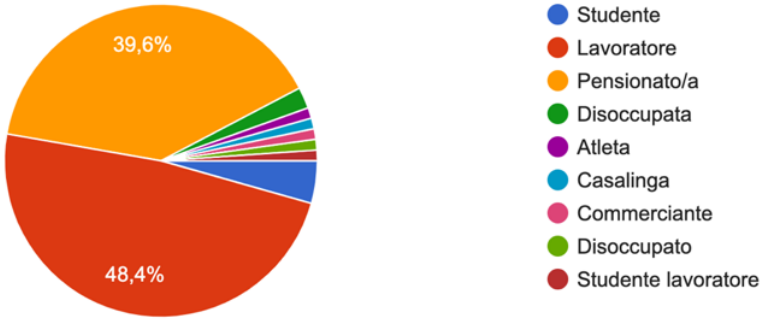
1) In che genere ti identifichi?
91 risposte



2) Di quale fascia di età fai parte?
91 risposte

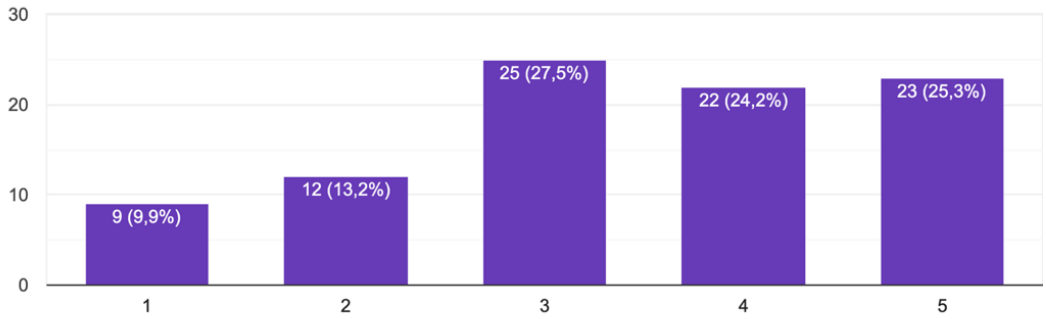


3) Qual è la tua occupazione?
91 risposte



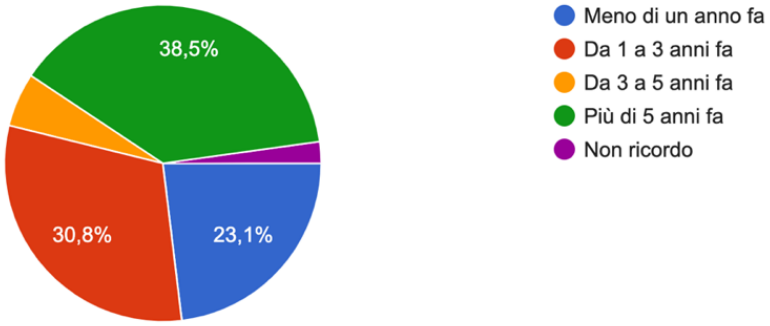
4) Da 1 a 5, quanto ritieni adeguata la tua conoscenza sulla patologia: trombocitopenia immune (ITP) e sui suoi relativi rischi correlati?

91 risposte



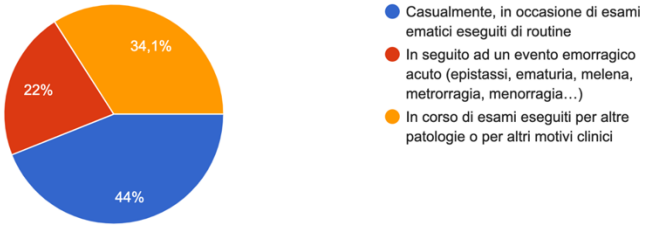
5) Quanto tempo fa hai ricevuto la diagnosi di ITP?

91 risposte



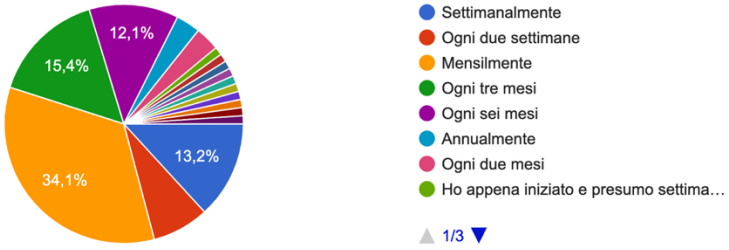
6) Come è stata fatta la diagnosi di ITP?

91 risposte



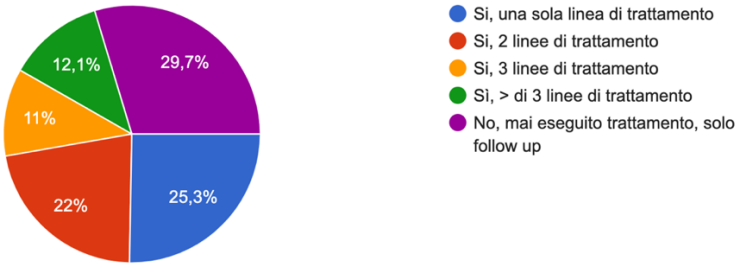
7) Ogni quanto effettui esami ematici e/o visite di controllo per l'ITP?

91 risposte



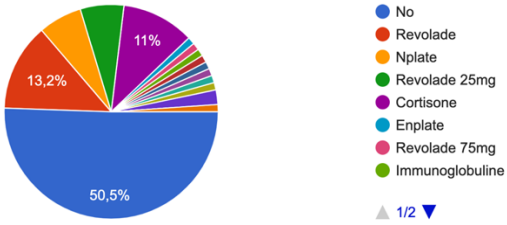
8) Hai mai ricevuto un trattamento terapeutico per l'ITP?

91 risposte



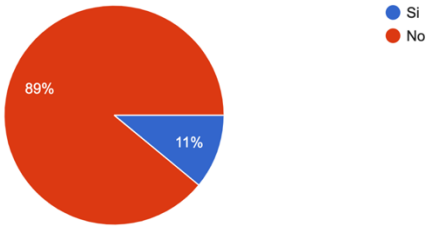
9) Al momento stai seguendo un trattamento per l'ITP?

91 risposte



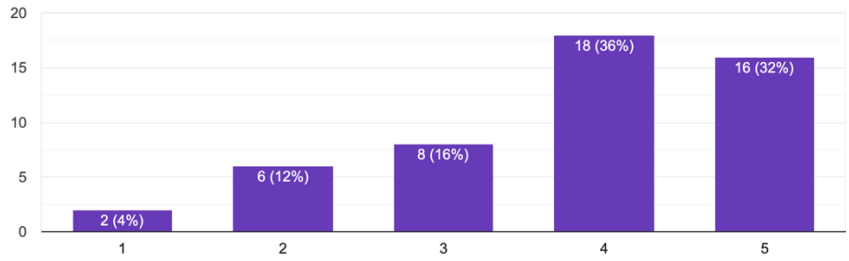
10) In passato, sei stato sottoposto a splenectomia come parte del trattamento per l'ITP?

91 risposte



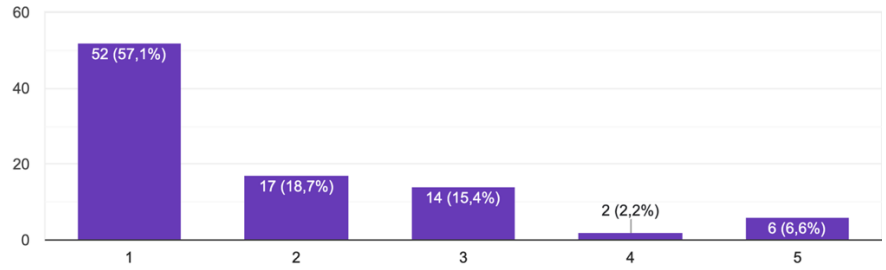
11) Se stai seguendo un trattamento per l'ITP, da 1 a 5, quanto sei soddisfatto dei risultati ottenuti fino ad ora?

50 risposte



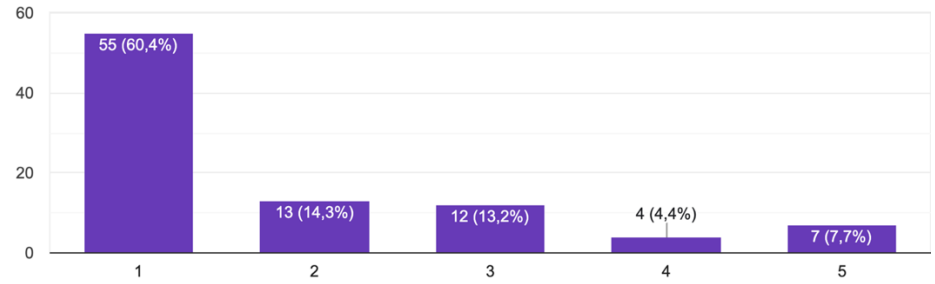
12) Da 1 a 5, quanto hai dovuto modificare il tuo stile di vita o le attività che svolgi a causa dell'ITP?

91 risposte



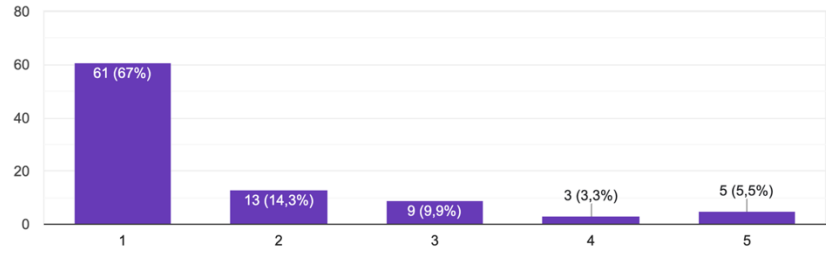
13) Da 1 a 5, quanto l'ITP ha influenzato la tua vita lavorativa o i tuoi studi?

91 risposte



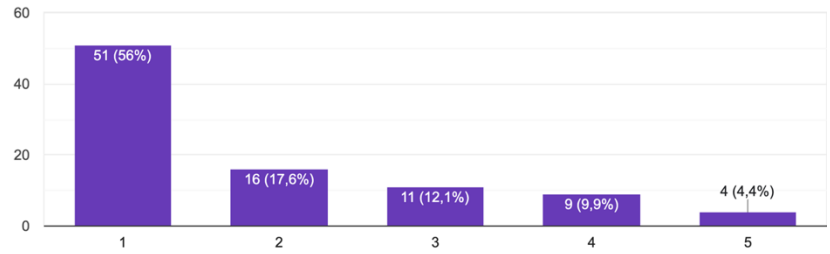
14) Da 1 a 5, quanto hai ridotto il tuo impegno lavorativo o scolastico/universitario a causa dell'ITP?

91 risposte



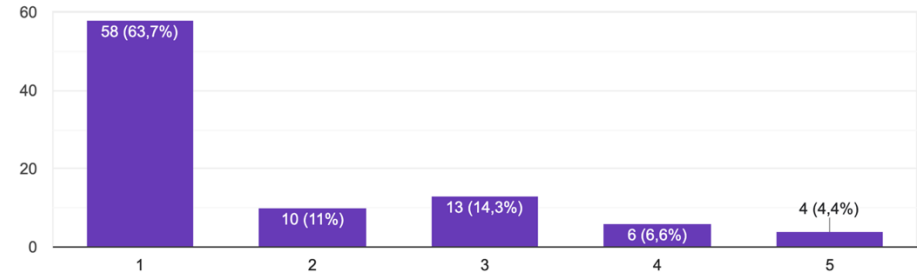
15) Da 1 a 5, quanto l'ITP ha influito sulla tua capacità di concentrazione nelle attività quotidiane?

91 risposte



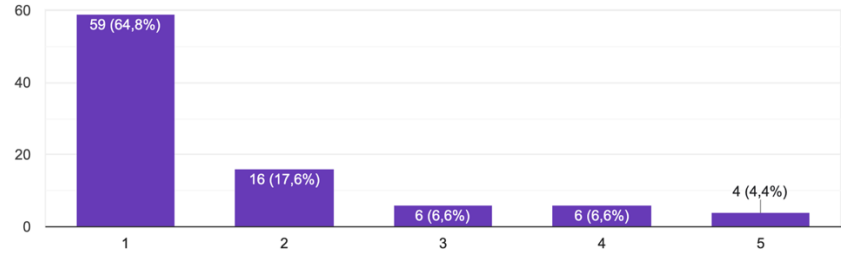
16) Da 1 a 5, quanto l'ITP ha impattato sulla tua vita sociale?

91 risposte



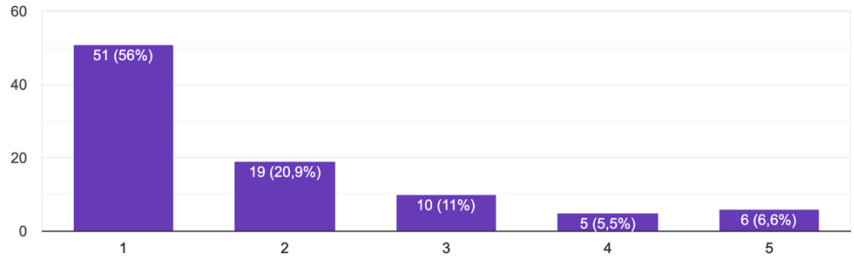
17) Da 1 a 5, quanto l'ITP ha impattato sui tuoi hobby?

91 risposte



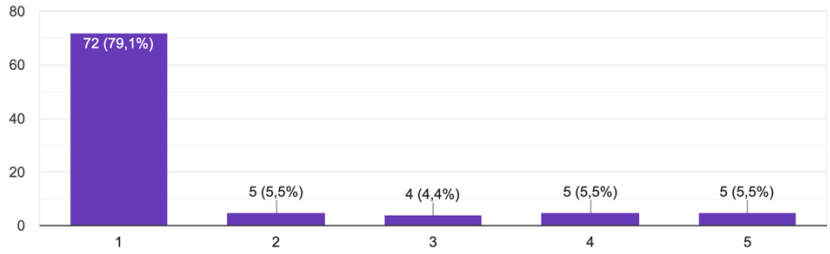
18) Da 1 a 5, quanto l'ITP ha compromesso la tua abilità nello svolgere attività fisiche normalmente?

91 risposte



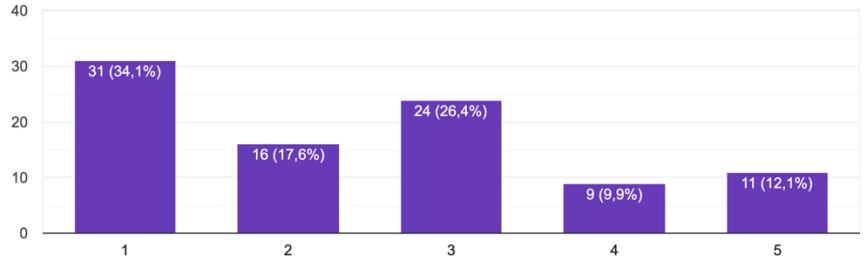
19) Da 1 a 5, quanto l'ITP ha impattato sulla tua vita sessuale?

91 risposte



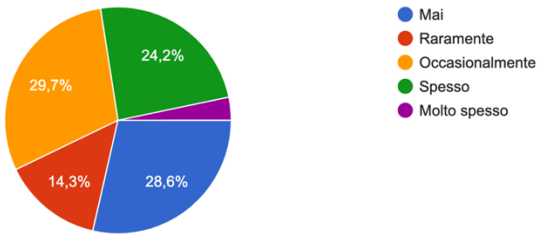
20) Da 1 a 5, quanto l'ITP ha influito sui tuoi livelli di energia e "voglia di fare"?

91 risposte



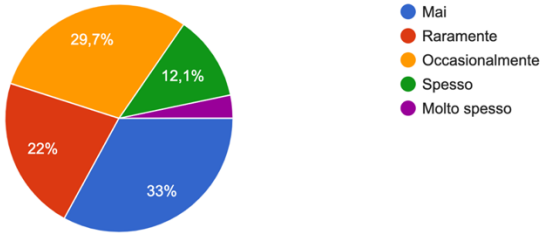
21) Con quale frequenza provi stress o ansia a causa dell'ITP?

91 risposte



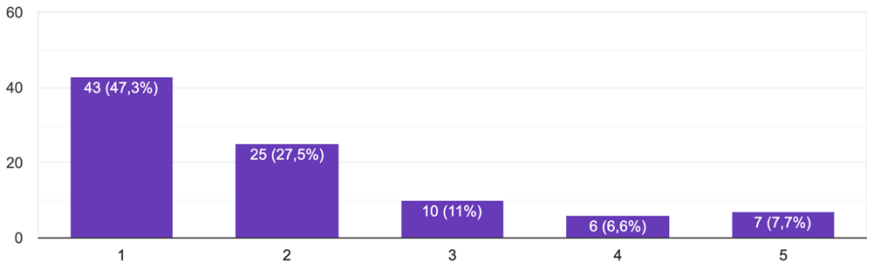
22) Con quale frequenza hai avuto sentimenti di tristezza, depressione o isolamento a causa dell'ITP?

91 risposte



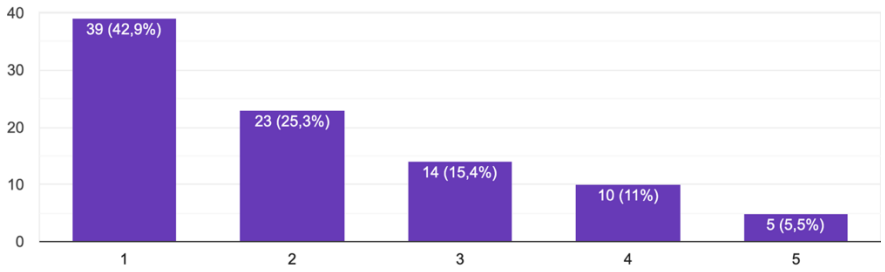
23) Da 1 a 5, quanto pensi che il trattamento per ITP abbia impattato negativamente sulla tua vita quotidiana?

91 risposte



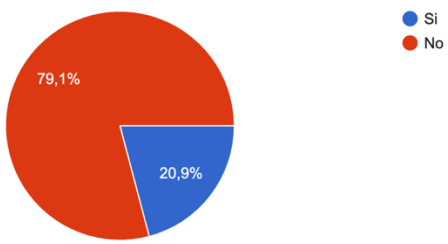
24) Da 1 a 5 quanto pensi che la patologia abbia influenzato la tua qualità complessiva di vita?

91 risposte



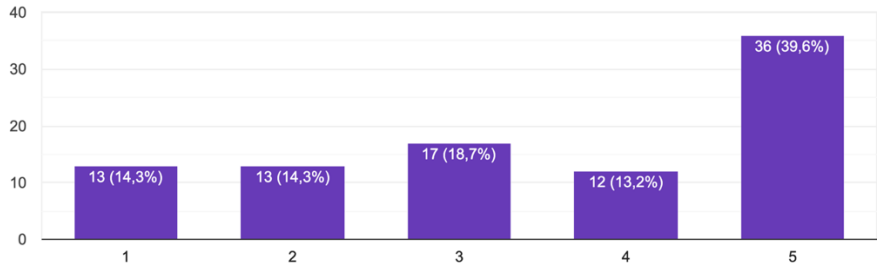
25) Riterresti utile per te un supporto psicologico mirato per affrontare lo stress correlato all'ITP?

91 risposte



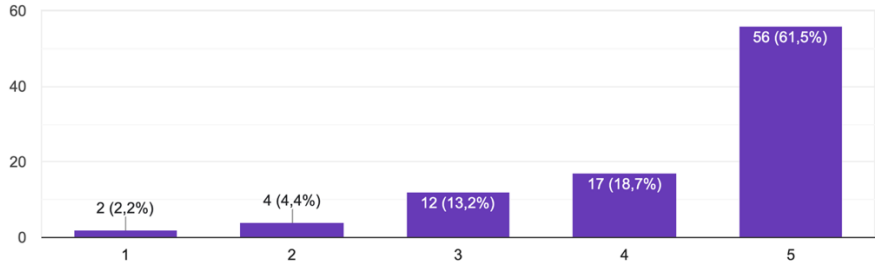
26) Da 1 a 5, quanto ti senti compreso dai tuoi parenti/conoscenti in merito alla tua condizione di salute?

91 risposte



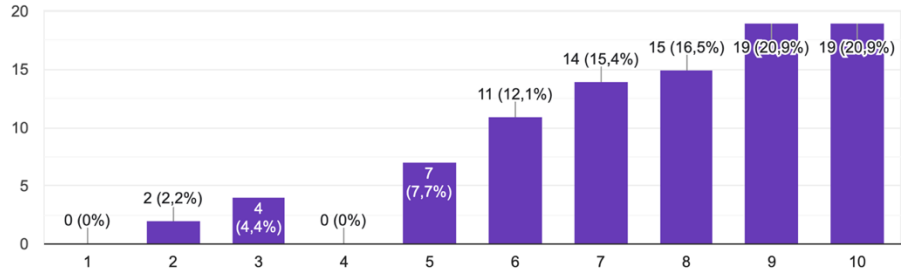
27) Da 1 a 5, quanto ti senti compreso dai professionisti che ti seguono per la patologia?

91 risposte



28) Da 1 a 10 come descriveresti nel complesso la tua esperienza da paziente rispetto alla patologia ITP?

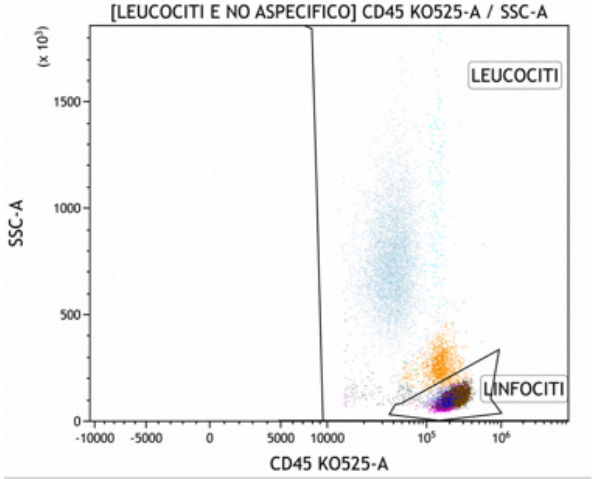
91 risposte



29) Le risposte che hai fornito sopra riguardano la tua situazione attuale. Queste percezioni sono rimaste invariate durante tutta la durata della tua malattia o ci sono state fasi in cui erano diverse?

91 risposte

Attachment 2. Pictures from peripheral blood immunophenotyping analysis of one of the patients from the study



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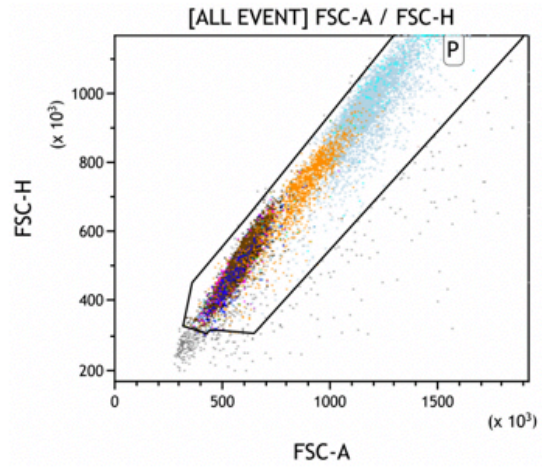

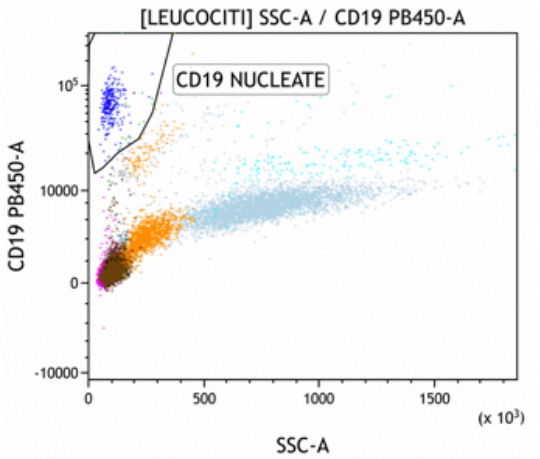

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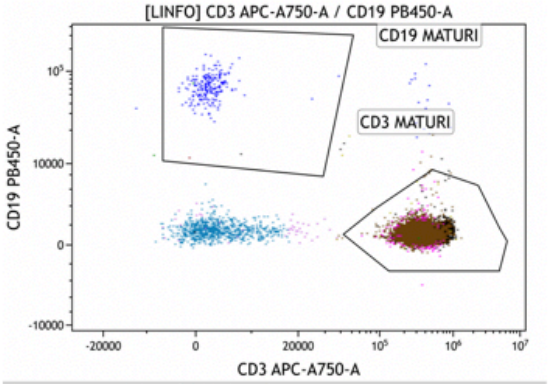

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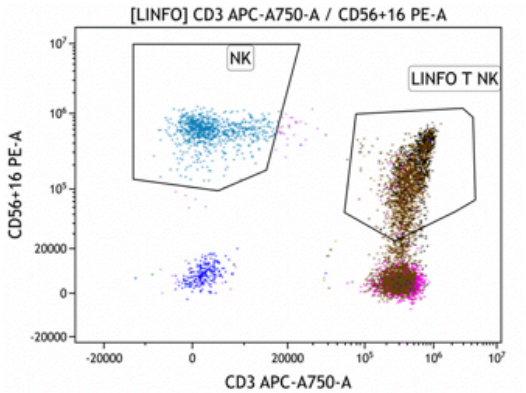
CD3 MATURI	84.7515	43.9101



Gate	%Gated	%GP Gated
LINFO T NK	32.2276	16.6972
NK	10.9873	5.6925