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THESIS

"Granulomatous Lymphocytic Interstitial Lung Disease in Common Variable Immunodeficiency: An Italian multicenter retrospective study"

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1. Abstract

Introduction

Common Variable Immunodeficiency (CVID) is the most common symptomatic Primary Antibody Deficiency (PAD) in adults and the most frequently diagnosed symptomatic Inborn Error of Immunity (IEI). Its clinical manifestations can be divided into two categories: infectious and non-infectious, reflecting the dual nature of the disease involving both immune deficiency and immune dysregulation. One of the most severe complications of CVID is Granulomatous Lymphocytic Interstitial Lung Disease (GLILD), a condition associated with poor outcomes and unclear pathogenesis.

Objectives

This study aimed to analyse GLILD in relation to mortality, pulmonary function, clinical phenotypes, and treatment. A key focus was developing a minimally invasive diagnostic approach by utilizing bronchoalveolar lavage fluid (BALF) analysis, reducing the need for more invasive diagnostic methods such as surgery or excessive radiological exposure. Additionally, we sought to identify clinical and immunological factors linked to a poorer prognosis.

Methods

We included patients from four Italian Referral Centers for IEI who had either histological or clinical-radiological evidence of GLILD. Patients with more severe manifestations, such as lymphoma, need for immunosuppressive therapy, or death, were categorized as having a poor prognosis (cases). In contrast, the remaining GLILD patients were placed in the good prognosis group (controls). Data for analysis were gathered through a retrospective review of medical records from both cases and controls.

Results

A total of 64 patients were included in the cohort. The majority were female

(72%). Lung and lymph node biopsies frequently showed lymphoid follicular hyperplasia (77% and 95%, respectively) and granulomas (69% and 63%, respectively). Genetic testing was performed on 12 patients, with mutations in TACI (33.3%) and CTLA-4 (25%) detected. All patients had immunoglobulin levels below the normal range at diagnosis (median IgG = 236 mg/dL; IgA = 10 mg/dL; IgM = 18 mg/dL), necessitating replacement therapy for the entire cohort. Extrapulmonary involvement was common (85%), with splenomegaly in 77%, hepatopathy in 31%, immune thrombocytopenia in 50%, and autoimmune hemolytic anemia in 20%. PET-CT scans showed lymph node involvement in the majority of cases (supradiaphragmatic 97%, subdiaphragmatic 85%), along with lung (85%) and spleen (61%) involvement. Cancer was diagnosed in 25% of patients, with half being hematologic malignancies.

Regarding lung involvement, pulmonary function tests revealed a reduced DLCO% (median 77% at the first test, 75% at the most recent). CT scans frequently showed lymphadenopathies (79%), nodules (75%), bronchiectasis (54%), and ground-glass opacities (50%). 63% of the cohort received specific treatment (steroids, rituximab, DMARDs), with 67.5% receiving rituximab and 52% responding to therapy. The overall mortality rate was 17%, with infections (46%), lymphomas (18%), and hepatopathy (9%) being the leading causes of GLILD-related deaths.

The case-control analysis yielded the following results: cases had a higher prevalence of females (82% vs. 58%; p=0.050), longer diagnostic delays (9 vs. 3 years; p=0.002), and more severe histological findings. Cases also had significantly lower IgG levels at diagnosis (189 vs. 298 mg/dL; p=0.027). In laboratory tests, the CD4+/CD8+ ratio was significantly lower in cases, both in serum (1.10 vs. 1.58; p=0.050) and BALF (1.10 vs. 4.13; p=0.004). Lung function tests showed lower DLCO% in cases (70.5% vs. 91.0%; p=0.002). Radiological findings showed that ground-glass opacities (71% vs. 19%; p<0.001) and bronchiectasis (71% vs. 6%; p=0.004) were more common in cases.

We developed a prognostic score based on IgG levels at diagnosis, diagnostic delay since CVID diagnosis, and DLCO% at the first pulmonary function test,

achieving a specificity of 95.2%, sensitivity of 75%, and an area under the curve (AUC) of 0.901.

Conclusion

This study confirmed several established characteristics of GLILD while also providing new insights, including factors that distinguish milder forms from more severe cases. The proposed prognostic score may help predict outcomes in GLILD patients. However, GLILD remains poorly understood, and further prospective studies with larger patient cohorts are necessary to establish more standardized, evidence-based approaches for diagnosing and managing this condition.

2. Introduction

2.1 Inborn Errors of Immunity (IEIs)

Inborn errors of immunity (IEIs) comprise a diverse group of nearly 500 disorders, most of which are characterized by specific genetic defects. Formerly known as Primary Immunodeficiency Disorders (PIDs), the terminology has shifted to emphasize the immune dysregulation that is a hallmark of these conditions[1]. The clinical spectrum of IEIs is broad, ranging from mild infections to more severe manifestations such as autoimmunity, autoinflammation, or cancer. Notably, this last aspect is a leading cause of mortality in patients with Common Variable Immunodeficiency (CVID)[2].

2.2 Primary Antibody Immunodeficiencies (PADs)

Primary antibody deficiencies (PADs) are a vast subgroup of IEIs which include a wide range of diseases. PADs are associated with a better long-term prognosis and a generally adult diagnosis, thus being the most diagnosed IEIs. The defect leading to these conditions can be B-cell intrinsic or extrinsic, but the aetiology is not completely understood. Even T-cell differentiation can be compromised, as T cells are deeply involved in B-cell activation[3].

The main components of this group include X-linked agammaglobulinemia (XLA), Hyper-IgM syndromes, Selective IgA deficiency or SIgAD, IgG subclass deficiency, unclassified antibody deficiencies (UAD) and Common Variable Immunodeficiency (CVID). and selective antibody deficiency with impaired specific response to polysaccharide antigens (SAD).

2.3 Common Variable Immunodeficiency (CVID)

Common Variable Immunodeficiency (CVID) is the most prevalent symptomatic PAD of adult [4] and the most diagnosed symptomatic IEI. It cannot be defined as a single disease, but rather as a heterogeneous group of disorders characterized

by hypogammaglobulinemia. In a big percentage of the cases, the underlying genetic defects are still unknown; however, nowadays, more and more often these defects are being identified, favoring a more tailored management.

The overall prevalence of this condition is set to be between 1 in 25,000 and 50,000[5,6]

According to the European Society for Immunodeficiencies (ESID) the criteria for the diagnosis of CVID include [7]:

At least one of the following:

- Increased susceptibility to infections
- Autoimmune manifestations
- Granulomatous disease
- Unexplained polyclonal lymphoproliferation
- Affected family member with antibody deficiency

AND marked decrease of IgG and marked decrease of IgA with or without low IgM levels (measured at least twice; <2SD of the normal levels for their age)

AND at least one of the following:

- Poor antibody response to vaccines (and/or absent iso-hemagglutinins)
- Low switched memory B cells (<70% of age-related normal value)

AND secondary causes of hypogammaglobulinemia have been excluded

AND diagnosis is established after the 4th year of life (but symptoms may be present before)

AND no evidence of profound T-cell deficiency, defined as 2 out of the following (y=years of life):

- CD4 numbers/microliter: 2-6y <300, 6-12y <250, >12y <200
- % naive of CD4: 2-6y <25%, 6-16y <20%, >16y <10%
- T cell proliferation absent

2.3.1 Clinical manifestations

The main features of CVID include recurrent bacterial infections (mainly involving upper and lower respiratory tract), often in association with autoimmunity, gastrointestinal involvement, splenomegaly, lymphoproliferative disorders and granulomatous organ infiltration. These symptoms can develop at an early age or during adulthood and the clinical phenotype is variable, so the diagnosis can be challenging, and the diagnostic delay can be significant[1]. (*See Table I and Figure 2*)[8].

Clinical manifestations of CVID	
Infectious manifestations	***Non-infectious manifestations***
Pneumonia (atypical and typical organisms)	Autoimmune hemolytic anemia
Airways disease	Granulomatous disease (pulmonary or systemic)
Otitis media	Cytopenia
Sinusitis	Splenomegaly
Conjunctivitis	Enteropathy
Enteritis	Polyarthritis
	Interstitial lung disease /GLILD
	Malignancy (lymphoma/MALToma)

Table I: Clinical manifestations of CVID[9].

2.3.2 Classifications

In consideration of the multifaceted clinical picture, two main classifications of CVID have been proposed: the Chapel classification and the Euroclass classification.

2.3.2.1 The Chapel phenotypic classification

The Chapel classification divides CVID patients into different categories according to their clinical phenotype [7,10]. It has been proposed in order to obtain a systematic division between the groups, to search for an eventual underlying common pathogenesis and to standardize the patients' management.

According to the last revision, the categories are:

- No other disease-related complications (described as "infections only" phenotype);
- Cytopenia (thrombocytopenia, autoimmune hemolytic anemia, neutropenia);
- Polyclonal lymphoproliferation (including persistent unexplained lymphadenopathy, lymphoid interstitial pneumonitis, noninfective granuloma);
- Unexplained persistent enteropathy.

Recently the last three groups tend to be frequently identified cumulatively as "complicated phenotype"

It has been reported that different groups have significantly different prognosis [11,12].

2.3.2.2 The Euroclass classification

The Euroclass classification analyses the link between flow-cytometric B-cell phenotyping and clinical manifestations (*see Figure 1*)[13]. It gets inspiration from the previous Freiburg classification[14] and Paris classification[15], improving the two methods.

Firstly, it separates patients with nearly absent B cells (less than 1%), identified as group B-, from a B+ group with a higher percentage of B cells. Then, the B+ group is divided according to the number of switched memory B cells: the subgroup with severely reduced smB cells (less than 2%) is called group smB-, while the other is defined as smB+. Thirdly, the smB- group is splat again in accordance with the level of transitional B cells, if more than 9% (Tr^{hi}) or less

(Tr^{norm}). Lastly, the smB+ and smB- groups are also classified accordingly with the $CD21^{low}$ B cell count (more or less than 10%).

The applications of this classification are several: for example, the B- group contains all patients with severe defects of early B-cell differentiation, while severely reduced switched memory B cells indicate a defective germinal center development. Moreover, correlations between subgroups and clinical manifestations have been searched: a reduction of smB may be associated with a higher risk for splenomegaly and granulomatous disease, whilst an elevation of $CD21^{low}$ B cells has seemed to be associated with splenomegaly and autoimmune disease; additionally, lymphadenopathy seems to be significantly linked with transitional B-cell expansion.

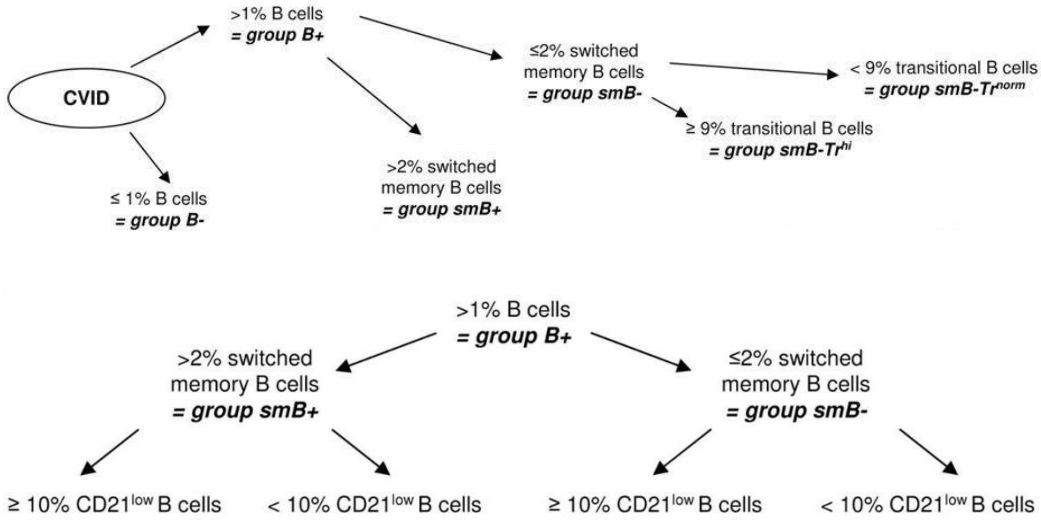

Figure 1: The Euroclass classification scheme.

2.3.3 Management

General management of CVID relies upon IgG replacement therapy (IgRT), either using Intravenous Ig (IVIG) or Subcutaneous Ig (SCIG)[16]. Both IVIG and SCIG have been proven to be effective in infection prevention, to be safe in terms of infusion-related adverse events [17,18] and to result in improved quality of life for affected patients. Regarding the dysregulated aspect of CVID, several immunosuppressive therapies are used to keep the condition under control.

Strategies can range from steroids to target therapy, based on the eventual underlying genetic defect.

2.4 Pulmonary Involvement in CVID

Respiratory disease is a significant contributor to both morbidity and mortality in affected patients. Complications include respiratory tract infections (RTIs), airway diseases, malignancies, and interstitial lung disease (ILD). Early diagnosis is essential, with pulmonary function tests (PFTs) and computed tomography (CT) being key tools in the initial evaluation and ongoing monitoring of CVID patients. The primary objective is to detect, assess, and quantify the extent of lung damage, providing a comprehensive understanding of the condition to guide treatment decisions.

Pulmonary complications, like other CVID complications, can be schematized into two different categories (*See Figure 2*)[19]:

- Infection-related (*e.g.* pneumonia or "degenerative patterns" as bronchiectasis or early COPD)
- Immune-mediated Interstitial Lung Diseases (*e.g.* GLILD)

It is also known that some conditions can be a result of both[20].

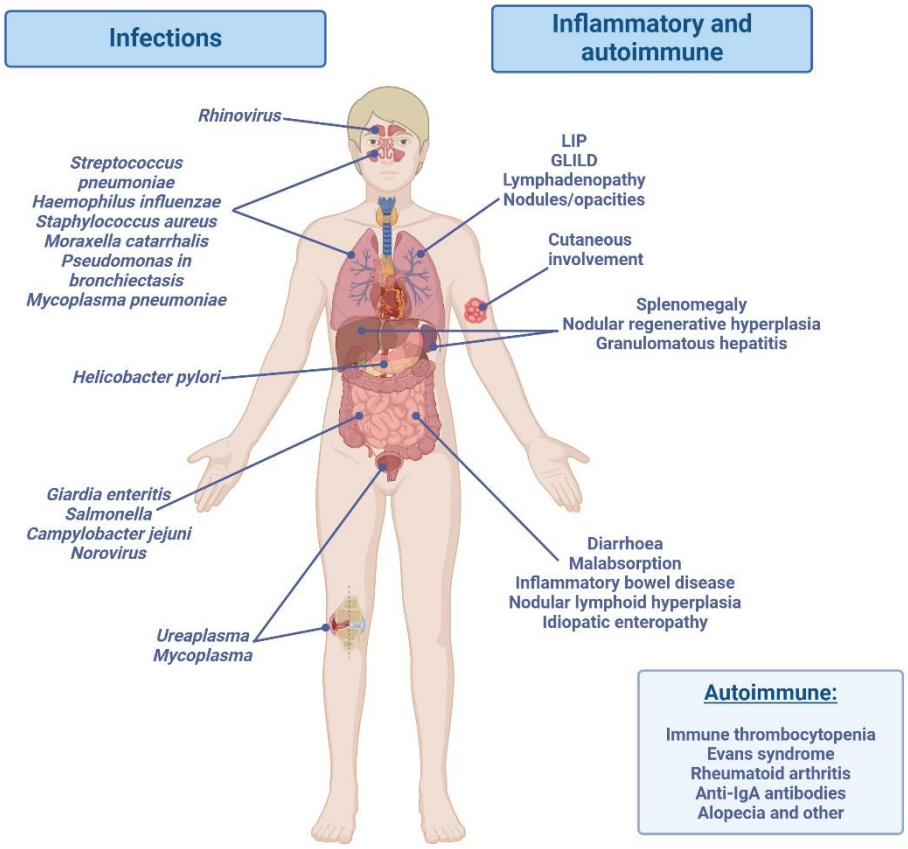

Figure 2: Infectious and non-infectious complications of common variable immunodeficiency[21]. *Created with BioRender.com.*

2.4.1 Respiratory tract infections (RTIs)

In the past decades, recurrent lower respiratory tract infections, particularly pneumonia, were a major issue for patients with CVID, often leading to high morbidity and mortality. However, advances in personalized immunoglobulin replacement therapy and antibiotic prophylaxis regimes[22] have significantly improved the management of these infections, making them less of a primary concern today.

Figure 3 displays the most prevalent respiratory pathogens, encompassing both bacterial and viral agents[23].

Opportunistic pathogens such as *Pneumocystis jirovecii* and *Cytomegalovirus* are less frequent but can be observed in CVID patients with low levels of $CD4^+$ T cells[24].

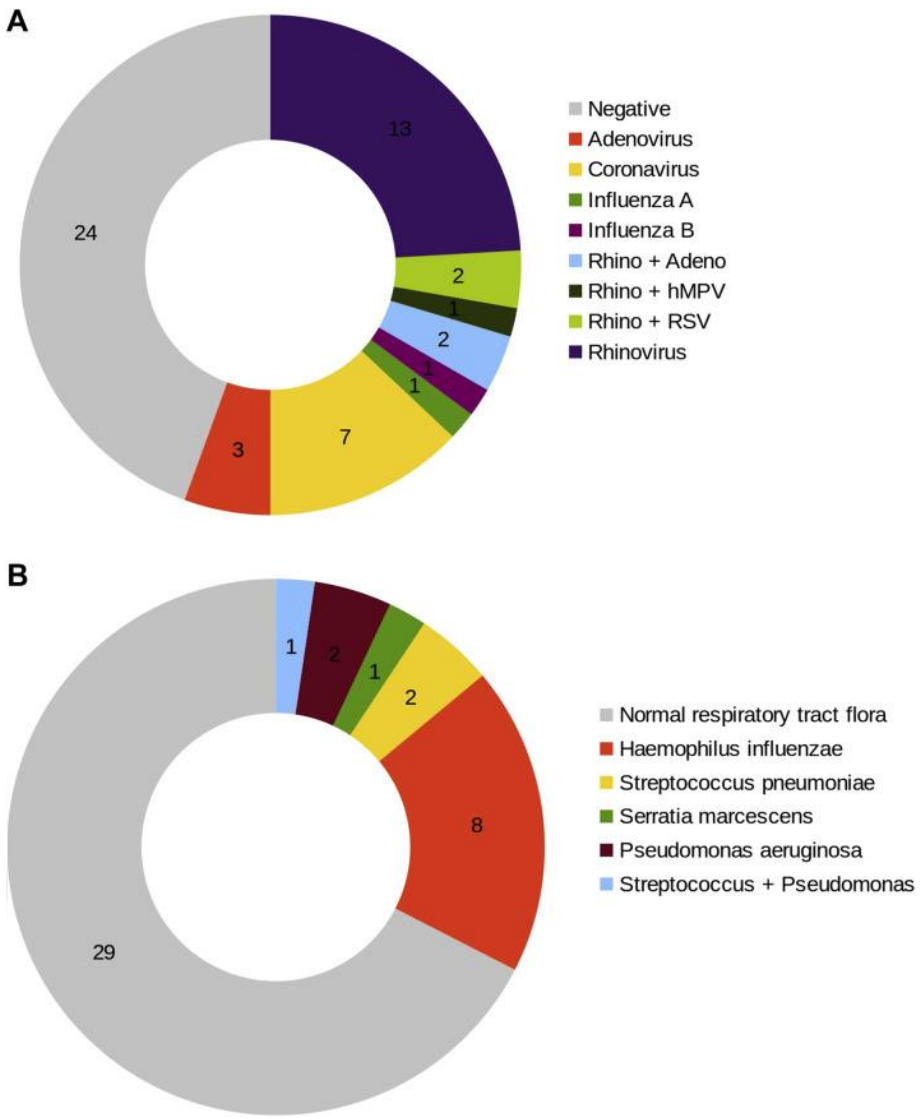

Figure 3: Pathogenic viruses (A) and bacteria (B) analysis from a sample of 69 patients with CVID[2]. Viral PCR was performed on nasopharyngeal swabs in 54 symptomatic patients (A), while bacterial culture was performed on spontaneously expectorated sputum in 43 patients (B).

2.4.2 Infection-related degenerative patterns

Analyzing the infection-related side, the recurrence of acute respiratory infections can lead to the development of chronic degenerative diseases (i.e. bronchiectasis and COPD)[25]

Bronchiectasis and COPD are the main infection-related chronic manifestations in CVIDs patients, being the leading causes of lung function decline[26].

Bronchiectasis is characterized by airway dilatation, resulting in a higher risk of developing repeated episodes of infection and inflammation, which worsen the airways' wall and leads to degenerative lung damage. This "vicious cycle" is enhanced in people with CVID, due to immune dysregulation (*see Figure 4)*[19].

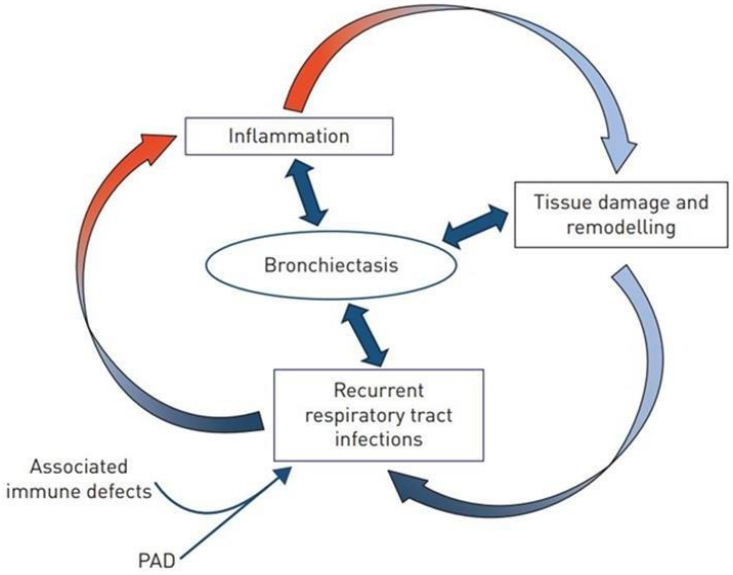

Figure 4: Pathogenesis of bronchiectasis and chronic obstructive lung disease in common variable immunodeficiency (CVID)

As aforementioned, the development of COPD is consequent to the persistence of active inflammatory response to recurrent infections[27]

2.4.3 Bronchiectasis/airway disease

Airways are deeply involved in CVID, and generally, this distress is manifested as bronchiectasis, mucoid impaction, and wall thickening. In particular, it has been observed that bronchiectasis and wall thickening are among the most common radiologic pulmonary abnormalities[28–30].

Bronchiectasis can affect more than half the CVID population [31], likely, this is deeply connected to recurrent lower respiratory tract infections (RTIs)[32], to the point that the severity of the bronchiectasis could be compared to the number of

prior RTIs. This may be the reason why patients presenting bronchiectasis are older or have longer treatment delays than patients without them[33].

Typical findings of distal mucoid involvement, resulting from repeated infections, on computed tomography (CT) are parenchymal centrilobular and tree-in-bud nodularity[34] (*see Figure 5*). Visual air trapping and airway thickening are usually associated with obstructive patterns in pulmonary function tests.

Progression occurs despite IVIG therapy with a mild impact of antibiotic prophylaxis.

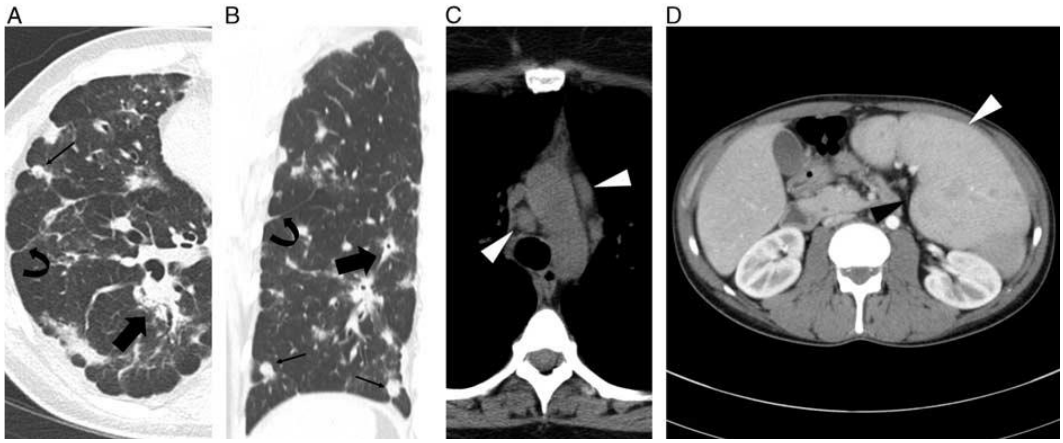

Figure 5: 34-year-old man with CVID and GLILD. A and B, Lung windows show septal thickening (curved arrows), nodularity (thin arrows), and consolidative opacities (block arrows) in a peri-broncho-vascular distribution. Axial soft tissue windows show mediastinal lymphadenopathy (arrowheads) (C) and splenomegaly (arrowheads) (D)[9].

2.4.4 Malignancy

Malignancies' incidence in CVID revolves around 2,5-16%, with the main character being non-Hodgkin lymphoma[35]. This condition may emerge from underlying non-clonal lymphoproliferative diseases, with an overall rate quite higher than the general population. Progression occurs despite IVIG therapy[36].

Concerning lung involvement, pulmonary lymphoma is characterized by mediastinal, hilar, and axillary lymphadenopathy on CT, with pulmonary nodules and masses in a perilymphatic distribution (*see Figure 6*)[9].

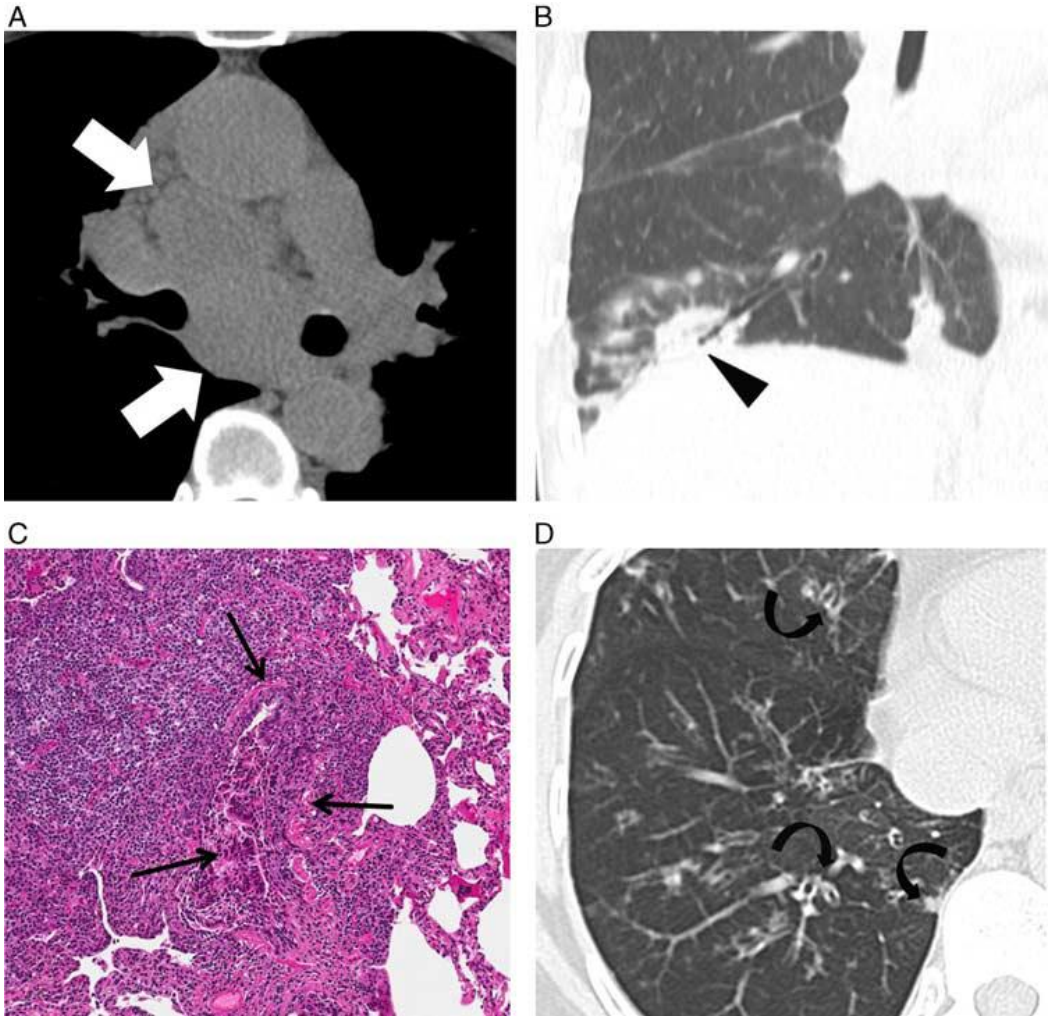

Figure 6: Lymphoproliferative disease in CVID. A, Axial CT in soft tissue windows shows extensive conglomerate mediastinal lymphadenopathy (block arrows). B, Coronal image of lung windows shows multiple multifocal pulmonary masses with air bronchograms (arrowheads) and surrounding satellite nodularity. C, Hematoxylin and eosin stain of a patient with pulmonary MALT lymphoma show lymphocytic infiltration, effacing normal architecture and destroying the adjacent bronchiole (thin arrows). D, Axial CT image of a patient with CVID complicated by MALT lymphoma shows peribronchovascular thickening and nodularity (curved arrows)[9].

2.4.5 Immune-mediated Interstitial lung disease (ILD)

Immune dysregulation in CVID is particularly pronounced, often presenting as Interstitial Lung Diseases (ILDs), which play a critical role in lung function decline among patients[4,37].

ILDs in CVID are typically difficult to diagnose due to their delayed symptoms, which often appear only after significant lung damage has occurred. Early detection through pulmonary function tests (PFTs) can reveal restrictive patterns and reduced diffusion capacity, leading to further examinations like the 6-minute

walk test or high-resolution computed tomography (HRCT) to uncover underlying issues[38].

ILDs can manifest in various forms, including follicular bronchiolitis, lymphocytic interstitial pneumonia (LIP), and non-specific interstitial pneumonia (NSIP). As treatment for infectious complications improves, non-infectious issues, particularly ILDs, are becoming a greater focus. In some patients, ILD may be the initial indication of CVID, emphasizing the importance of early detection[39].

Two primary patterns characterize ILD in CVID: granulomatous and lymphocytic, which can coexist and complicate diagnosis[16]. Histologically, the granulomas are typically non-necrotizing and can vary in appearance[39,40]. Again, biopsies can recognize an overlap of findings, mainly attributable to lymphocytic interstitial pneumonia (LIP), organizing pneumonia (OP) and granulomatous disease. While the lungs are most commonly affected, CVID is a systemic condition, with granulomas potentially present in other organs.

2.5 Granulomatous Interstitial Lung Disease (GLILD)

2.5.1 Definition

Granulomatous Lymphocytic Interstitial Lung Disease (GLILD) is a rare complication of CVID. It has been defined as "a distinct clinic-radio-pathological ILD occurring in patients with [common variable immunodeficiency disorders], associated with a lymphocytic infiltrate and/or granuloma in the lung, and in whom other conditions have been considered and where possible excluded" [41].

GLILD is a condition mainly affecting the lungs of CVID patients, but it may have a wider spectrum of distribution, characterizing a multi-systemic disease: besides the lungs, the main sites involved appear to be lymph nodes, liver, and spleen, leading to lymphadenopathy, hepatomegaly, and splenomegaly.

It has been observed that in some cases GLILD appears as the first manifestation of CVID[42].

2.5.2 Incidence and prognosis

It has been estimated that GLILD can be diagnosed in around 20% of CVID cases[41].

As it concerns prognosis, GLILD is burdened by a poorer outcome than CVID. When observing the Kaplan-Meier survival plot below (*see Figure 7*), it is possible to note that the median survival in Groups 1, 2 and 3B taken together, representing CVID non-GLILD patients, is significantly higher than the median survival of Group 3A, composed of GLILD patients (precisely, 28.8 to 13.7)[43].

Moreover, in the 3A group, GLILD is associated with dyspnoea, splenomegaly, restrictive pulmonary physiology and consolidation, and ground-glass or reticular radiographic abnormalities. These complications may have a key role in worsening the prognosis and the quality of life of these patients.

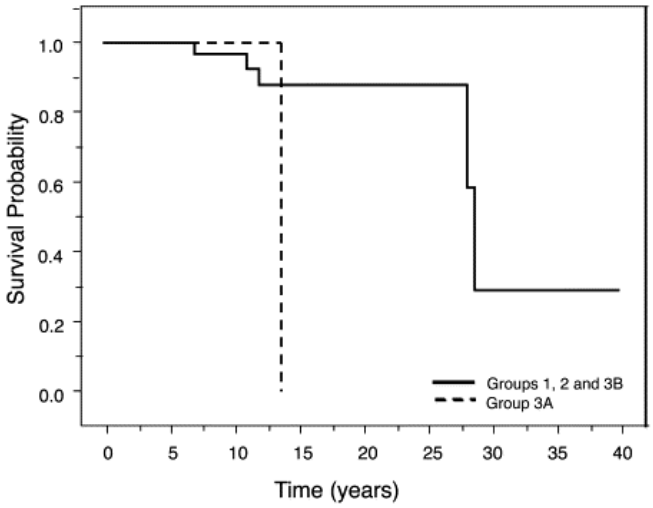

Figure 7: Kaplan-Meier survival plot demonstrating differences between the 2 groups of patients (GLILD vs non-GLILD). The median survival of 28.8 years in groups 1, 2, and 3B (solid line) *is compared with the median survival of 13.7 years in group 3A* (dashed line; P < *.001). There is no statistical difference in survival between groups 1, 2, and 3B. Time is from the date of CVID diagnosis.*[43]

2.5.2.1 GLILD and COVID-19: impact on prognosis and quality of life

During the SARS-CoV-2 pandemic years, our research group analyzed various clinical aspects of patients with CVID who contracted the infection, as well as their vaccine response, demonstrating that in patients with a complicated phenotype, particularly those with autoimmunity and chronic lung disease, the vaccine response was poorer compared to healthy individuals and uncomplicated CVID patients, even after multiple vaccine booster doses. [44–46]

Particular attention was given to the risk of hospitalization and mortality among CVID patients with a complicated phenotype and chronic lung disease (especially GLILD), compared to the general population and uncomplicated CVID patients. Patients with end-stage lung disease related to GLILD showed a higher rate of hospitalization, especially those already undergoing oxygen therapy, due to exacerbation of lung disease and higher risk or superinfections (GLILD vs non-GLILD p=0.002), and notably a higher mortality rate compared to patients without lung complications.[47,48]

Additionally, we showed that patients with lung involvement and GLILD more frequently required targeted antiviral treatments and monoclonal antibodies, precisely because showing a highter risk for hospitalization and mortality

(regression models showed that GLILD was a risk factor for hospital admission (OR 4.40, 95%CI 1.51-12.86, p<0.007) whereas end-stage lung disease was a risk factor both for exitus (OR 44.22, 95%CI 3.65- 536.1, p=0.003), and disease severity (OR 23.14, 95%CI 3.11- 181.74, p = 0.002). [48] Considering that, our group evidences that tailored targeted therapy with both antivirals and monoclonal antibodies in this kind of patients had a positive effect on reducing the risk of hospitalization (OR 0.12, 95%CI 0.043-0.340, p <0.001).[49]

This risk also inevitably impacted the quality of life of these patients, who more frequently experienced concern for the future and a fear of illness during the pandemic era. [50,51]

Finally, we observed a higher risk of developing long-term post-infection symptoms from SARS-CoV-2 (long COVID), with manifestations such as arthralgia, myalgia, fatigue, and shortness of breath, particularly in patients with a complicated phenotype, autoimmune cytopenia, COPD, GLILD, and bronchiectasis. Specifically, the development and prolonged persistence of long COVID were significantly correlated with female sex and a complicated phenotype in multivariate analysis. [52]

2.5.3 Pathogenesis

GLILD pathogenesis is not completely understood yet. However, there have been several studies analyzing if there may be some facilitating or promoting factors.

Firstly, the role of germline CTLA-4 haploinsufficiency has been emphasized in promoting lymphoproliferation, lymphocytic infiltration of non-lymphoid organs, autoimmune cytopenia, and B cell abnormalities, including the accumulation of CD21low B cells[53]. Cytotoxic T lymphocyte antigen-4 (CTLA-4) is an inhibitory receptor that is constitutively expressed by FoxP3+ regulatory T cells (Tregs), which play a critical role in maintaining immune tolerance. It is also expressed by activated T cells, where it serves a suppressive function. CTLA-4 has been extensively utilized to modulate immune responses in autoimmune diseases and cancer, through CTLA4-Ig fusion proteins and neutralizing CTLA-4 antibodies. When mutated, CTLA-4 expression can be significantly reduced,

leading to a severe loss of immune tolerance and the development of infiltrative autoimmune disease.

T cells are deeply interested in GLILD: raised serum markers of T cell activation and exhaustion have been detected (*e.g.* sCD25, sTIM-3, IFN-γ, TNF)[54], suggesting that these cells may have a key role in the pathogenesis of this condition.

On the other side, it is *not* just the T cell phenotype that has been detected as altered in GLILD patients.

It has already been studied that most CVID patients, regardless of GLILD status, have a higher frequency of $HLADR^{+}CD4^{+}$ T cells, $CD57^{+}CD8^{+}$ T cells, and $CD21^{low}$ B cells when compared to healthy controls. However, *only* in CVID/GLILD patients, it has been observed an alteration of TCR/BCR signalling in the activated lymphocyte populations[55].

Also, B cell dysregulation is a key element in the pathogenic pathway of GLILD[56]. One potential mechanism involves the increased activity of B cell activating factor (BAFF). BAFF, a cytokine regulated by interferon gamma (IFN-γ) and part of the STAT1 signalling pathway, plays a crucial role in B cell activation and survival. It is a member of the TNF ligand superfamily and is primarily produced by monocytes, macrophages, neutrophils, and dendritic cells. BAFF binds to three receptors on B cells: the BAFF receptor (BAFFR), transmembrane activator and CAML interactor (TACI), and B cell maturation antigen (BCMA)[57]. These receptors are essential for B cell activation, proliferation, and survival. BAFFR, for instance, is critical for the survival and maturation of peripheral B cells, while TACI inhibits B cell expansion, induces IgG and IgA class switch recombination, and promotes plasma cell differentiation and survival. Elevated BAFF levels have been found in both the serum and lungs of CVID patients with interstitial lung disease (ILD)[58]. Lymphoid hyperplasia in CVID-ILD is thought to result from BAFF-driven B cell stimulation (*see Figure 8*)[58]. Additionally, recent studies suggest that TACI signalling may contribute to immune dysregulation and be linked to the development of certain autoimmune conditions, such as systemic lupus erythematosus (SLE)[59 60].

Another risk factor which may be included in high IgM serum levels: it seems that this might correlate with a higher activity of pulmonary B cells, leading to increased follicle formation and sped-up ILD development[37,58].

So far, it has been discussed the role of humoral and cell-mediated adaptive immunity. Nevertheless, innate immunity is also essential in the pathogenesis of GLILD. One of the main histopathological features of GLILD is the sarcoid-like non-caseating granuloma, composed of giant cells and epithelioid histiocytes. These cells descend from the monocyte-macrophage lineage through the mTORC1 pathway, which many studies have suggested as the main driver of the granuloma formation in sarcoidosis[61]. The mechanism seems to be similar in GLILD, as it has shown the reported efficacy of Sirolimus, a mTORC1 inhibitor, in the treatment of two GLILD patients [62].

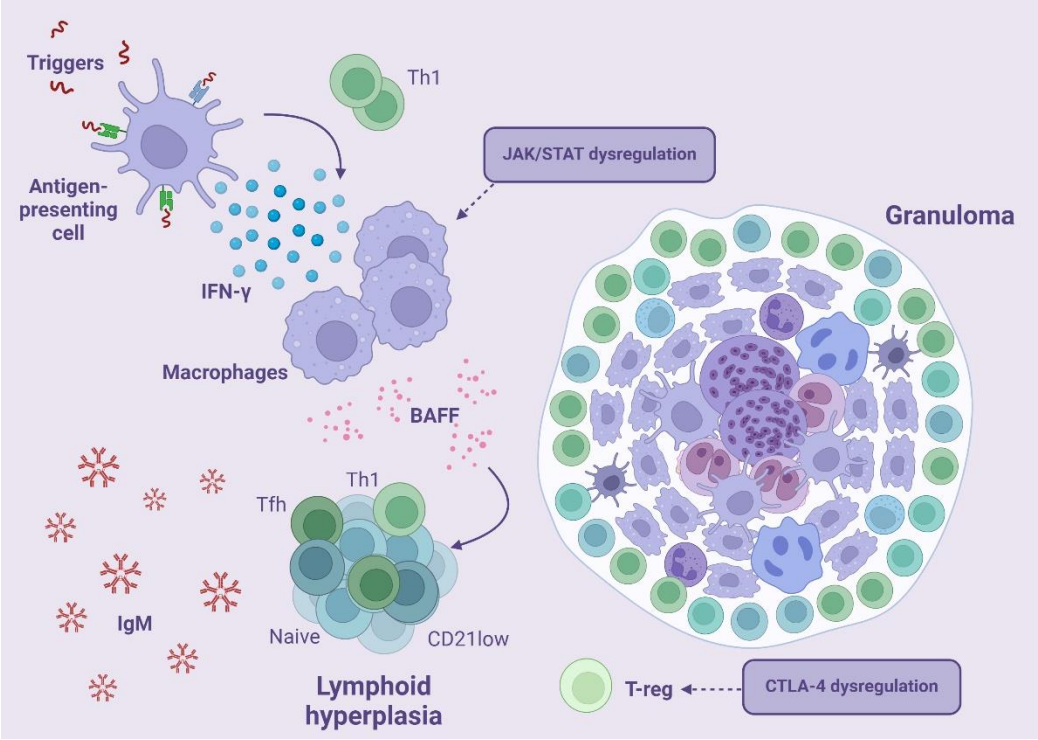

Figure 8: GLILD pathogenesis. The first trigger seems to be linked to chronic antigen exposure, eventually with predisposing environmental factors. Then, antigens are captured by antigen-presenting cells (APCs), which communicate with macrophages and T helper cells through interferon-gamma (IFN-γ). Macrophages and other cell populations can produce B cell activating factor (BAFF), which encourages lymphoid hyperplasia formation and IgM production; these cells can also be subjected to dysregulation, mainly regarding the JAK/STAT pathway. CTLA-4 is another factor that can facilitate lymphoproliferation and loss of tolerance. The final step is

represented by the sarcoid-like non-caseating granuloma, which is a typical finding in GLILD patients. Created with BioRender.com.

2.5.4 Clinical manifestations

Symptoms are usually vague (*e.g.* cough, shortness of breath) and the onset is insidious. A significant percentage of patients may be asymptomatic. Moreover, clinical manifestations often appear when the condition is in a late stage, and the lungs are already compromised. The prognosis is worse than in CVID non-GLILD patients, as aforementioned[43].

Some symptoms may be correlated to GLILD's collateral conditions: cytopenia is usually frequent in GLILD patients, in particular immune thrombocytopenia (ITP); splenomegaly is also quite a common finding.

2.5.5 Biomarkers and possible predictors

Many studies have been analysing GLILD biomarkers as predictors of disease onset or progression (*see Figure 9*)[58,63].

Clinical manifestations can be of use, but they usually appear in later stages. Splenomegaly and widespread lymphadenopathy are the main findings, but also polyarthritis may have a role [64]. Other symptoms may be expressions of an underlying cytopenia, whose main percentage is represented by Immune Thrombocytopenia (ITP) but also autoimmune haemolytic anaemia (AIHA).

On the other hand, laboratory biomarkers may also be important predictors: elevated IgM, decreased IgG or IgA, cytopenia, and low B or T cell markers are the main factors [35,54,55].

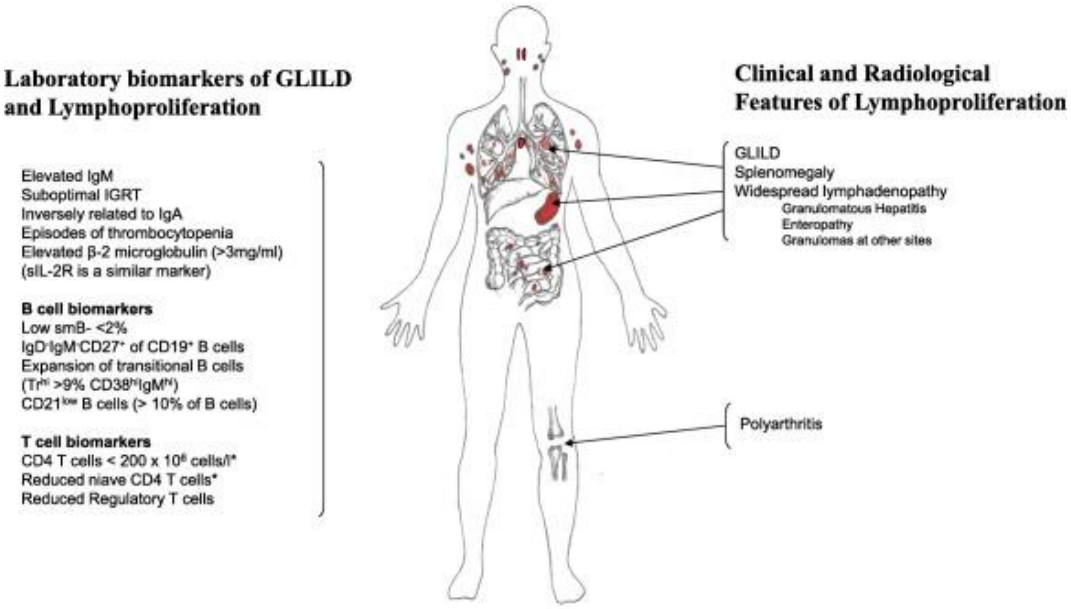

Figure 9: Clinical and laboratory risk factors for granulomatous lymphocytic interstitial lung disease (GLILD)[67]

As aforementioned, HLADR+CD4+ T cells, CD57+CD8+ T cells, $CD21^{low}$ B cells and BAFF are deeply involved in the pathogenesis of CVID and GLILD[54].

Additionally, serum markers of T cell activation and exhaustion (*e.g.* sCD25, sTIM-3, IFN-γ, TNF) may have a role in defining the development of the disease[55].

Given these biomarkers and clinical findings, many studies have tried to define which parameters have a diagnostic or prognostic significance.

Unfortunately, the question is still open, as there has not been an agreement yet.

A 2016 study conducted on thirty-four GLILD patients concluded that hypersplenism and polyarthritis seem to be strong risk factors for GLILD[63].

However, a more recent study, based on twenty-six cases, claimed that splenomegaly, history of ITP or AIHA and percentage expansion of $CD21^{low}$ B cells may be useful to identify high-risk groups[64]. Moreover, a diagnostic algorithm- was proposed (*see Figure 10*).

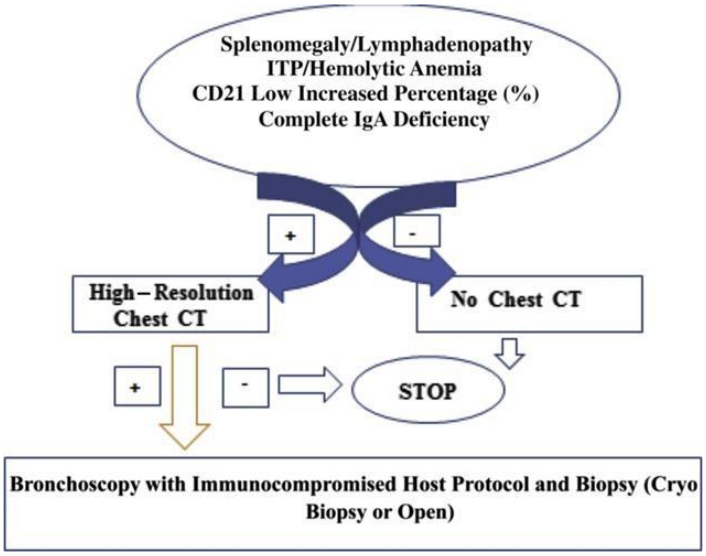

Figure 10: Proposed algorithm for the evaluation of CVID patients at risk for GLILD[64].

Then Our group suggested that a diagnostic tool for early identification of GLILD should have included a combination of two clinical parameters (splenomegaly and autoimmune cytopenia), one lung function index (DLCO %) and one immunologic variable ($CD21^{low}$ B cells%)[66] (*see Figure 11*).

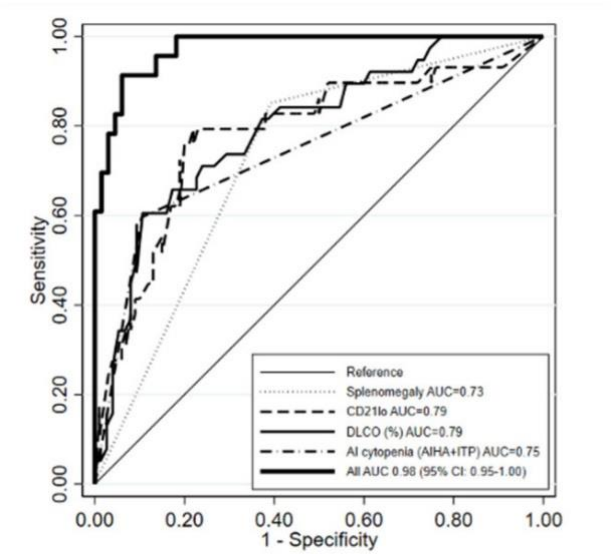

Figure 11: ROC curve and area under the curve (AUC) of a proposed GLILD diagnostic score[66].

A recent study analyzed fifty patients from a referral Center for primary immunodeficiencies, identifying several key predictors: lymphadenopathy, splenomegaly, Baumann's GLILD score (a radiological score assessing the number of pulmonary lobes with specific alterations), and CD8+ cell count[68].

Another radiological scoring system, the Hartmann score, has also been proposed. While it shares similarities with the Baumann score, it differs slightly and is primarily based on a cystic fibrosis-CT scoring method. The Hartmann score has shown slightly better reproducibility compared to the Baumann method[69].

However, there remains a lack of evidence-based guidelines, and larger studies are needed to establish clearer diagnostic and prognostic criteria.

2.5.7 Diagnosis

Given the not entirely clear pathogenesis, the often-vague clinical presentation with late-onset, and the laboratory characteristics that are not always straightforward, there are currently no universally accepted guidelines for the diagnosis and management of the disease.

At the moment, according to a document of the British Lung Foundation/UK PID network [43], histologic evidence of GLILD is needed for diagnosis, but new studies are reconsidering biopsies' crucial role in the diagnostic process[41,56]

At the present time, diagnosis revolves around three main approaches[70]:

- Imaging (*e.g.* HRCT, PET-CT)
- Pulmonary function tests (PFTs)
- Histology (*e.g.* lung biopsy)

Analysis of broncho-alveolar lavage (BAL) is also frequently performed, primarily to exclude infections[9]. The analysis of BALF can be implemented in the next years, tempting to study also the B and T cell profile and the cytokine expression[71,72].

Radiology is the most used method of investigation, as it first raises suspicion of GLILD. The differential diagnosis for GLILD is represented by infections,

lymphoma, sarcoidosis, cryptogenic organizing pneumonia (COP), lymphoid interstitial pneumonia (LIP), and other interstitial lung diseases.

PFTs are also widely used, showing mainly a restrictive pattern and reduced gas transfer.

Currently, the gold standard for diagnosis is surgical lung biopsy (open lung or video-assisted thoracoscopic, VATS)[9,41], a procedure associated with a high risk of mortality and morbidity in already fragile patients, often resulting in reduced survival and quality of life. There is no consensus on trans-bronchial biopsies, considering the frequent scarcity of the collected material which makes detailed histological analysis difficult.

2.5.7.1 Imaging

HRCT is an essential tool, as morphologic alterations generally occur earlier than functional dysregulation.

The main HRCT findings of CVID include diffuse bronchiectasis, bronchial wall thickening, atelectasis and air trapping [73], but the most peculiar aspects of GLILD are the presence of solid and semisolid nodules, diffuse ground-glass, fibrosis areas, and hilar and/or mediastinal lymphadenopathy (*see Figure 12*)[21,63,64].

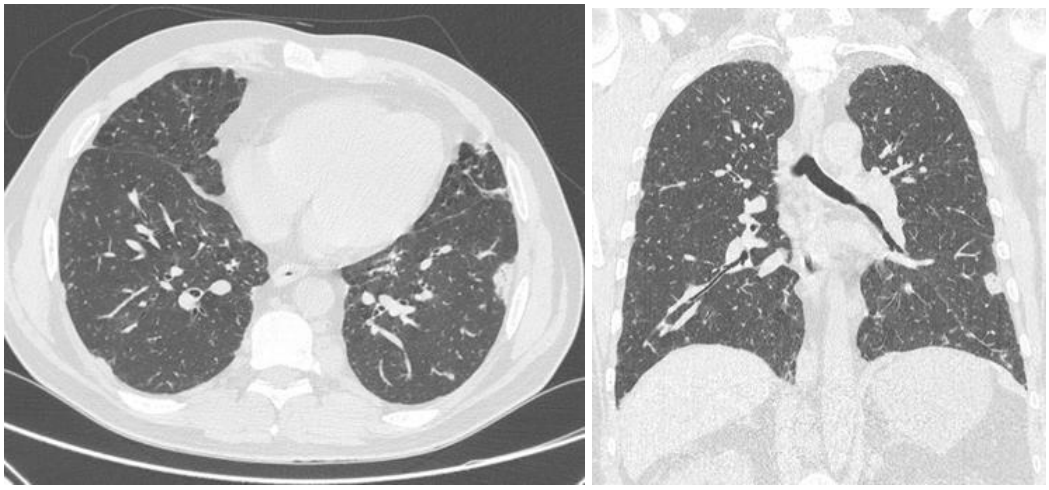

Figure 12: Typical HRCT pattern of one of our GLILD patients. Imaging from patients included in the present study.

The radiological pattern needs to be scrupulously observed by a trained Radiologist, in order to conduct an accurate differential diagnosis. The main conditions that can mimicry GLILD's nodules are lymphomas and sarcoidosis. Sarcoidosis, in particular, is frequently compared to GLILD, because both of them present non-necrotizing systemic granulomas and have preferential involvement of lungs and lymph nodes. Moreover, both sarcoidosis and GLILD are based on profound immune dysregulation, probably due to an impairment in antigen clearance[76]. However, there are some features that allow for discriminate: nodules in GLILD patients are usually larger (>3 cm) and have a predominant basal distribution, while sarcoidosis is characterized by micronodules, frequently confined to the upper lobe (*see Figure 13*).

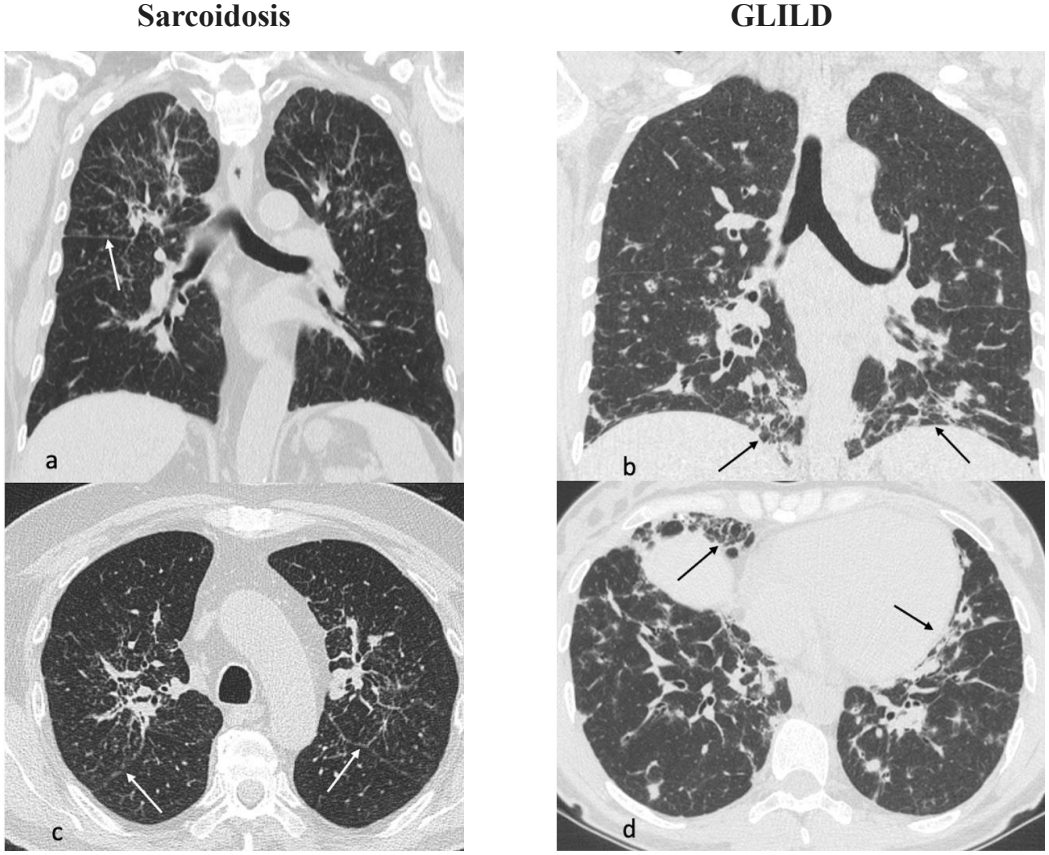

Figure 13: HRCT of our two patients, one with sarcoidosis ***(a, c)*** *and one with GLILD* ***(b, d)****. It appears clearly that sarcoidosis has a prevalent medium-upper involvement, with multiple peri lymphatic nodules (*white arrows*) and perihilar fibrosis. On the other hand, GLILD has a lower distribution, with consolidations and nodules; reticulations (*black arrows*) with some signs of fibrosis can also be seen*[76].

Recent evidence supports the possible role of lung magnetic resonance imaging (MRI) in diagnosing and monitoring lung disease in GLILD patients[70]. This would be an important improvement, reducing the patient's exposure to ionizing radiation.

Another important emerging tool for GLILD's monitoring and follow-up is fluorodeoxyglucose (FDG)-positron emission tomography-computed tomography (PET-CT). This technique is generally used to observe the development of the condition on a systemic scale and to assess the response to treatment. It can detect both morphological and functional changes, but its strength is in the possibility to compare the metabolic activity of the same area over time, to eventually identify new inflammatory areas. Moreover, it can give a clearer picture of the extrapulmonary dissemination of the pathology, addressing an eventual therapy (*see Figure 14*).

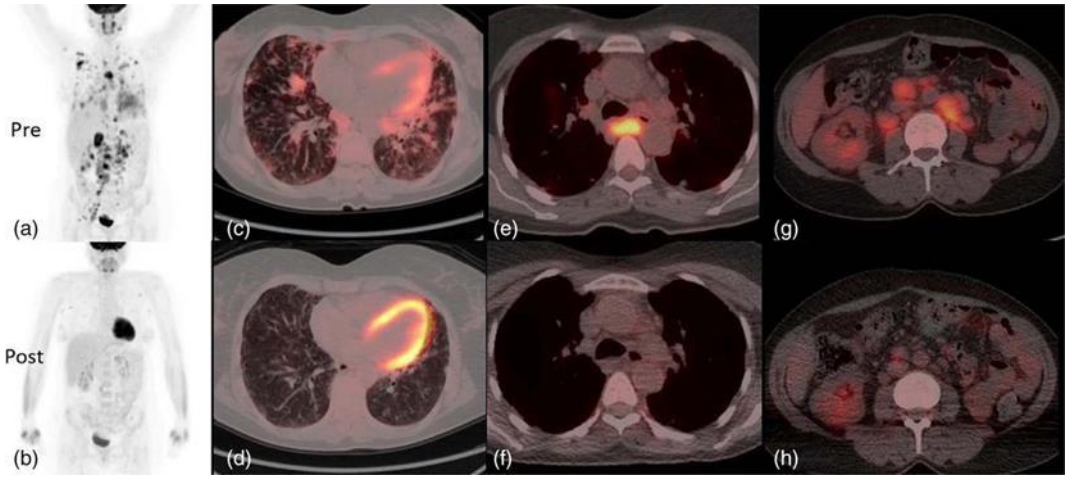

Figure 14: 2-[(18)F]-fluoro-2-deoxy-d-glucose positron emission tomography and computed tomography (FDG PET-CT) pre- and 3 months post- specific GLILD immunosuppressive treatment with rituximab and mycophenolate[67].

In conclusion, even if histology is always required, imaging can be reliable in determining the severity of the condition: it has been observed that patients with progressive GLILD, defined by deteriorating pulmonary function, have significantly greater pathology on pulmonary CT and FDG-PET-CT scans as compared to patients with stable disease[66,77]. This means that there is actually a correlation between the morphologic and functional aspects of the disorder.

2.5.7.2 Pulmonary Function Test

Pulmonary function tests (PFTs) are assessment tools widely used to analyze the impact of GLILD on lung functionality, especially because it is a non-invasive and easily reproducible test. Usually, they include spirometry, measurement of the diffusing capacity of the lung for carbon monoxide (DLCO), and assessment of static lung volumes (*e.g.* total lung capacity, TLC, and residual volume, RV).

However, the results can be very different from one patient to another and vary from normal to severely impaired: GLILD patients' PFTs can present a restrictive pattern, an obstructive pattern, or a mixed result. On the other hand, gas transfer is usually low in most of the cases. [37]

As recently highlighted by our research group, CVID-GLILD patients showed a greater decline in spirometric parameters compared to non-GLILD-CVID in a 10-year follow-up, especially in terms of TLC and DLCO which decreased respectively from the 16^{th} to 5^{th} percentile and from the 16^{th} to the 3^{rd} from the first to the last spirometry performed. [78]

2.5.7.3 Histology

Biopsies are a valuable diagnostic tool for GLILD, providing crucial information on lymphocytic infiltration and/or granuloma formation. As the final step in the multi-stage diagnostic process, histological analysis plays a critical role in confirming the disease, making it essential that the procedure is performed accurately.

The gold standards are the open lung biopsy and the video-assisted thoracoscopic surgery (VATS) biopsy[41,70], but they are invasive and present several side effects. In particular, patients who undergo these procedures may develop infections, prolonged air leakage, persistent pain, or even death (under 2% of cases)[79]. These risks are higher in patients with severely compromised lung function [80].

Transbronchial biopsy, on the other side, may be used to exclude different diagnoses, such as infections and lymphomas. The main limit of this procedure is that it is not sensitive enough to give acceptable results for a GLILD diagnosis, due to the small sample size.

A biopsy of another affected site can be considered, as it may be less invasive and less hazardous for the patient. Lymph nodes, for example, are a safer alternative.

When analyzing the biopsies material, GLILD has a very heterogeneous histologic presentation (*see Figure 15*). Peribronchiolar and interstitial lymphocytic infiltration, sarcoid-like noncaseating granulomatous hyperplasia, and organizing pneumonia are consistent features. Lymphoid hyperplasia is deeply connected to the granulomas, either because the granuloma is surrounded by lymphocytic infiltrate or because the granuloma itself spreads out around the reactive follicle. Extensive organizing pneumonia and interstitial fibrosis with architectural remodeling have also been seen in a significant proportion of patients[40,81].

Lymph nodes in GLILD usually present ill-defined germinal centers, eventually with an altered polarisation and infiltrated $CD8^+$ T-cells. Class-switched plasma cells (PCs) are usually absent or severely reduced, and if present, rarely found in the medulla of the lymph node[82]. This may be an indicator of germinal center failure.

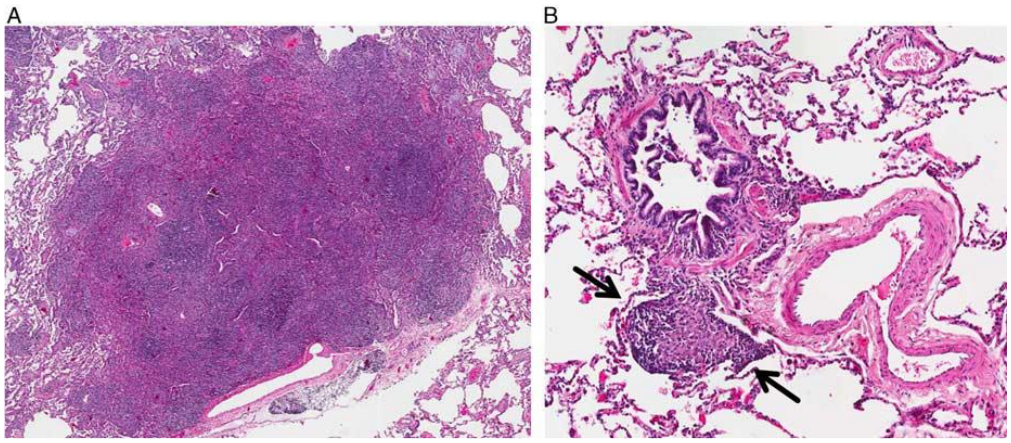

Figure 15: A, Haematoxylin, and eosin stain of GLILD shows nodular areas of lymphoid hyperplasia. The underlying architecture is preserved despite the density of the lymphocytic infiltrate.
*B, In the setting of granulomatous infiltration, a non-necrotizing granuloma (*arrows*) is immediately adjacent to the broncho vascular bundle*[9].

The goal set for the future is to remove biopsies from the diagnostic setup (or at least prefer less invasive biopsy techniques) and the reason behind this can be

found in the aforementioned side effects. Nevertheless, at the moment it is impossible to have a clear diagnosis of GLILD without a biopsy[41].

2.5.7.4 Bronchoalveolar lavage (BAL)

The analysis of bronchoalveolar lavage fluid (BALF) is recommended for microscopy, bacterial, mycobacterial, and fungal culture[41].

On the contrary, flow-cytometric analysis is not always reliable or reproducible, as it has not been proven a significant role in the diagnostic process. Anyway, it can be performed to gain more information, and the technical analysis has to be implemented in the next years, including cytokine expression to better define the pathogenic background of the disease.

The main features of GLILD that have been studied at BALF analysis so far are the expansion of T cells and B cells and, in particular, of $CD21^{low}$ B cells, a subpopulation that has been recently studied for its potential diagnostic [71,72].

2.5.8 Management

There is currently no systematic protocol for managing and treating GLILD, as there are no official guidelines available. Although numerous studies[83] have been conducted, a consensus on the most effective treatment strategy has not been reached.

Various publications report on small-scale studies involving monotherapy with glucocorticoids or other immunosuppressants, rituximab alone or in combination with azathioprine, abatacept, or hematopoietic stem cell transplantation (HSCT). Treatment response rates differ significantly, and mortality rates, particularly among immunocompromised patients, remain high. Given the systemic nature of the disease, a multidisciplinary approach is essential. Additionally, since patients may exhibit diverse organ involvement, therapies should be as personalized as possible.

Firstly, before starting a specific therapy, IgG replacement therapy is strongly suggested, to optimize the immunoglobulin levels of the patients. The injection can be intravenous (IVIG) or subcutaneous (SCIG). The subcutaneous route presents some differences from the intravenous route therapy: more frequent and

smaller doses, lower peaks and higher troughs, more patient autonomy, decreased systemic adverse effects[18], and the lack of a requirement for vascular access[84]. Both the procedures seem to enhance survival and reduce severe and invasive infections[85] but is also well-known the "immunomodulatory" effect of immunoglobulins that can take part in the modulation of autoimmune and inflammatory background of CVID and GLILD[86–88].

Adjunctive therapies, such as Azithromycin prophylaxis[89] and pulmonary rehabilitation, in some cases, have a great role in controlling GLILD respiratory complications and deterioration.

It is not clear which may be the appropriate timing for specific immunosuppressive treatment initiation, but the choice should be based on the presence and severity of symptoms and the grade of lung impairment.

The first line of treatment, after the Ig replacement therapy, is represented by oral steroids[90,91]. They have a great impact in improving gas transfer in GLILD, nevertheless patients often relapse. Mycophenolate may be used as a maintenance therapy, as it seems to be associated with long-term effectiveness and permits weaning of corticosteroids[91].

The second line of treatment is generally agreed to include immunosuppressive drugs such as azathioprine, mycophenolate, and rituximab[41,92,93].

Azathioprine and mycophenolate are part of the family of disease-modifying antirheumatic drugs (DMARDs), which may be an option to reduce the usage of corticosteroids. Other exponents are cyclosporin, hydroxychloroquine and methotrexate. Azathioprine's mechanism of action, in particular, works directly against T cells, which appear to have an undiscovered role in the pathogenesis.

Rituximab, on the other hand, affects exclusively the B cells, interacting with their CD20. Symptom burden and quality of life have significantly improved in most of the treated patients, lung function has increased, and lung CT scan findings have decreased [94]. Therefore, rituximab can be considered a widely effective option[95]; in fact, it has been observed that relapses are less frequent in patients treated with rituximab-containing treatment regimens if compared to

corticosteroid monotherapy[93]. However, there is currently no data on the long-term efficacy and side effects of rituximab and other second-line therapeutic options[92].

A combination therapy of rituximab and azathioprine has been proposed, to target B and T cells at the same time (*see Figure 16*). This therapy has been studied to be effective, determining an improvement of both lung function and radiographic findings[96].

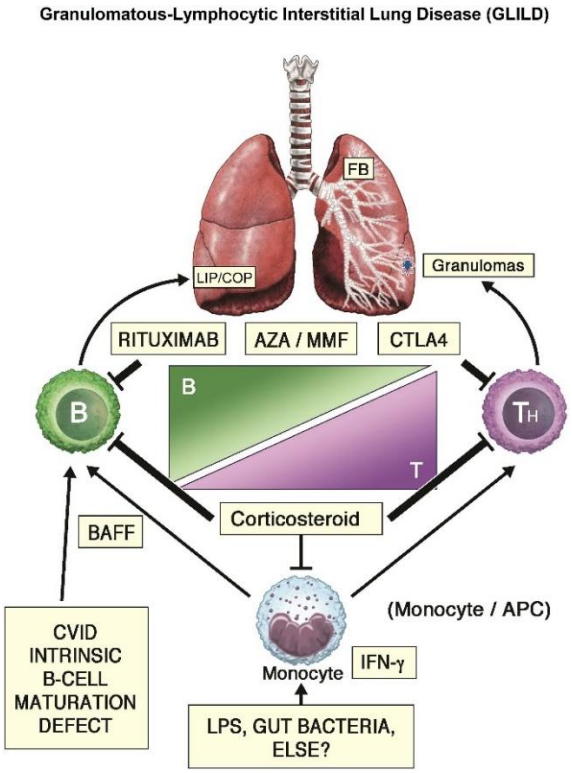

Figure 16: Cooperation between B and T cell inhibitors in the treatment of GLILD. GLILD in CVID seems to be driven by increased IFN-γ production, which stimulates macrophages, via STAT1 signaling, to secrete more BAFF. Together with CVID-intrinsic B-cell maturation defects, this seems to lead to pulmonary lymphoproliferation. Conversely, the role of local T cells in the exact etiopathogenesis of GLILD remains obscure, but a short course of glucocorticoid therapy, rituximab monotherapy, or rituximab plus azathioprine (AZA) or mycophenolate (MMF) treatment seems at least partly effective[97].

Other possible options of treatment may be represented by tacrolimus, sirolimus, abatacept, and anti-TNF agents (*e.g.* Infliximab[98]). Also, Belimumab, an anti-BAFF, might be a promising alternative treatment approach, considering the aforementioned role of BAFF in the pathogenesis of the disease [99].

Hematopoietic stem cell transplantation (HSCT), in addition, is a promising alternative to the pharmacological treatment of GLILD, because it has the potential to resolve the underlying pathology, not just to contain symptoms. Anyway, the risks for this procedure are high[93].

Finally, in the later stages of GLILD, when the lung function has critically decreased and the symptoms are severe, the only remaining option is lung transplantation[100,101].

3. Aims of the study

GLILD is a relatively obscure condition, with no large cohort studies conducted and limited knowledge regarding its clinical behavior and appropriate management.

This retrospective study aims to achieve the following objectives:

- To characterize the clinical behavior, survival rates, and mortality of GLILD patients in an Italian multicentric cohort.
- To analyze pulmonary function trajectories in GLILD patients and identify parameters that significantly correlate with the development and prognosis of the disease.
- To enhance understanding of the clinical manifestations of GLILD by distinguishing between the various phenotypes in which the disease may present.
- To provide a comprehensive overview of the different diagnostic and therapeutic strategies and their impact on patient outcomes.

4. Materials and methods

4.1 Study population

We considered all patients with GLILD followed by four Italian Referral Centres for adult primary immune deficiency: AOU Policlinico Umberto I, Sapienza University of Rome, Ca' Foncello Hospital in Treviso, Federico II University Hospital in Naples, and Cagliari University Hospital.

The Inclusion criteria were:

- At least 18 years of age;
- Histologic or clinical-radiologic diagnosis or suspicion of GLILD (given the absence of official diagnostic guidelines);

- Availability in the clinical records of the reports of laboratory and radiological parameters regarding GLILD diagnosis and follow-up (IgG, IgA, and IgM concentrations at diagnosis, eventual cancer or decrease);
- Signature of the informed consent.

On the other hand, no specific exclusion criteria were defined.

GLILD patients were further divided into 2 groups. A subgroup of patients with a **negative prognosis** was selected to be part of the "**case group"**. These patients presented at least one of these characteristics in addition to the inclusion criteria:

- Need for immunosuppressive therapy other than steroids (*e.g.* DMARDs, Rituximab);
- Occurrence of lymphoma;
- Death occurred for GLILD-related complications.

All the GLILD patients who did not meet the criteria were enrolled in the positive prognosis group (**control group**).

4.2 Data collection

This study was conducted via a retrospective review of medical records of cases and controls from the referral centers of Rome, Treviso, Naples, and Cagliari. The data collected for each patient, when available, were:

- Demographic information such as age, sex, year of first symptoms, and year of diagnosis (both symptoms and diagnosis of either CVID or GLILD were analyzed);
- The eventual environmental exposition to chronic antigens (smoke, occupational exposition);
- The value of IgG, IgA, and IgM at diagnosis and last available follow-up;
- The antibody response to tetanus vaccine and pneumococcal vaccine;
- Presence of comorbidities: splenomegaly (with eventual splenectomy),

bronchiectasis, autoimmunity (with particular attention to ITP, AIHA, and other cytopenia), cancer (either hematologic or solid organ);

- The type (IvIg or ScIg) and the dose of immunoglobulin replacement therapy at diagnosis and last available follow-up;
- Eventual histologic diagnosis of GLILD, in particular distinguishing lung biopsy from lymph node biopsy or other organs;
- Presence of extrapulmonary involvement (*e.g.* hepatopathy, lymphadenopathy);
- The analysis of B cell subtype according to Euroclass flow-cytometric classification (considering the percentage and the absolute number of CD19, CD27+IgM-IgD- switched memory B cells, CD27-IgM+IgD+ naïve B cells, CD27+IgM+IgD+ marginal zone B cells, $CD21^{low}$ $CD38^{low}$ activated B cells, CD38++IgM++ transitional B cells and CD38+++IgM- plasmablasts).

  Although the study is multicentric and the methodology is not standardized, a shared protocol was used for the identification of cell subtypes. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation and immediately frozen and stored in liquid nitrogen until use or the cells were analyzed immediately after isolation. B cell subsets were identified based on the expression of CD19 BV786, CD27 BV510, CD24 BV711, and CD38 BV421, IgM APC markers by flow cytometry from BD Bioscience. T cell subsets were identified based on the expression of CD3 Alexa700, CD4PE-Cy5, and CD8BUV395. Stained PBMC samples were acquired on FACs;
- The analysis of differential cell count and lymphocyte subtypes on BALF (considering the percentage of macrophages, neutrophils, eosinophils, and lymphocytes; of this last population, CD3+, CD19+, the CD4+/CD8+ ratio were analyzed, with eventually additional B cell subtypes according to Euroclass flow-cytometric classification).

  Despite the multicentric nature of the study and the lack of a standardized

methodology, a common protocol was employed for the identification of cell subtypes. The BALF was filtered and centrifuged at room temperature. The supernatant was divided into aliquots and frozen at -80°C. The pellet or cellular sediment was resuspended, and the cells were washed with sterile saline. Any contaminating red blood cells in the BALF were lysed. Subsequently, after another centrifugation at room temperature, the cells were stained with the same antibodies used to stain the PBMCs.

- Genetic tests' results (in particular Next-generation sequencing (NGS): allows for the simultaneous analysis of multiple genes to identify pathogenic mutations; Sanger sequencing: used to confirm specific mutations in known genes; Whole Genome Sequencing (WGS): to determine the entire DNA sequence of an organism by analyzing all of its chromosomes);
- HRCT evaluation, considering these parameters: ground glass opacities, nodules, bronchiectasis, lymphadenopathy;
- Organ involvement based on ^{18}FDG-PET-CT (in particular supradiaphragmatic and subdiaphragmatic lymph nodes, lungs, spleen, and liver glucosidic hyper-caption);
- Every pulmonary function test ever performed by the patient, in particular, the following functional lung parameters: FEV1, FVC, TLC, and DLCO;
- Immunosuppressive therapy (*e.g.* DMARDs, steroids, rituximab);
- Eventual death (and its cause);

The study was conducted according to the Helsinki Declaration and current Italian regulations and approved by the local Ethical Authority (Study 1342 CE Marca).

4.3 Statistical analysis

Data were collected in a Microsoft Excel database and analyzed using the software programs "Jamovi 2.3.28" and "GraphPad 10."

Descriptive statistics were applied to assess demographic characteristics, clinical manifestations, humoral and radiological findings, treatment options, and mortality.

Due to the relatively small sample size, non-parametric tests were used. Quantitative variables were summarized as medians with interquartile ranges, while qualitative variables were presented as percentages. Continuous variables were compared using the Mann-Whitney U test, and categorical variables were compared using Fisher's exact test. To evaluate changes over time within the same group, the Wilcoxon matched-pair signed-rank test was employed.

A survival analysis was conducted to generate a Kaplan-Meier curve for the mortality rate. For pulmonary function, z-scores and percentiles were used to illustrate the distribution and compare the sample to the general population. Logistic regression analysis was performed to explore potential associations between variables and GLILD prognosis, and ROC curves were used to assess the prognostic value of individual parameters.

5. Results

5.1 Description of study population

The population we analyzed included 64 patients, followed by the four Referral Centres:

- 28 patients from Ca' Foncello Hospital in Treviso (one of which was referred by Ancona University Hospital);
- 23 patients from AOU Policlinico Umberto I, Sapienza University of Rome;
- 9 patients from Federico II University Hospital in Naples;
- 4 patients from Cagliari University Hospital.

All of these patients fulfilled the above-mentioned inclusion criteria.

Among them, we identified 38 patients (59% of the sample) to be enrolled in the "negative prognosis group" (**case group)**.

All the patients who did not fulfill the criteria (26 patients, representing 41% of the sample) were enrolled in the **control group**. These patients appeared to have a milder phenotype, so they were considered the "positive prognosis group".

5.1.1 Demographic characteristics

The demographic characteristics of the whole cohort are summarized in the following *Table II,* expressed as median with interquartile ranges.

Table II: Demographic characteristics of the whole cohort

Median and interquartile range of age, age at diagnosis, age at first symptoms and diagnostic delay of respectively CVID and GLILD in the sample.

Age	50.5 (38.75-60.25)
Age at first symptoms of CVID	30 (16.75-37.25)
Age at first symptoms of GLILD	39 (29-48)
Age at diagnosis of CVID	38 (26.25-46.75)
Age at diagnosis of GLILD	44 (34-56)
Diagnostic delay of CVID	5 (2.75-13)
Diagnostic delay of GLILD	2 (0-7.75)

The general population did not present surprising characteristics compared to the Italian CVID population, except for the sex distribution, which was relatively unbalanced towards the female sex (*see Figure 17*). We compared the data with the sex distribution from the whole CVID cohort of the participating centers, which appeared to have a more balanced sex distribution (F=56.7%, M=43.4%).

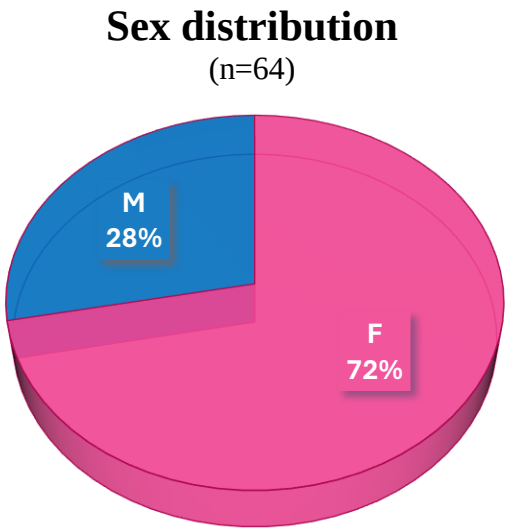

Figure 17: Sex distribution of the whole GLILD sample.

Focusing on antigenic exposure, 14 patients were former smokers, while one was an active smoker. This group had a median of 235 cigarette packs/year. Moreover, 6 patients were considered to have had professional exposure in their lives, mainly due to jobs in the mechanic field.

5.1.2 Biopsies and histological findings

Of all our patients, 29 underwent at least one biopsy, whose sites are summarized in the following *Figure 18*. In total, we obtained 43 biopsies, given the fact that some patients had more than one biopsy.

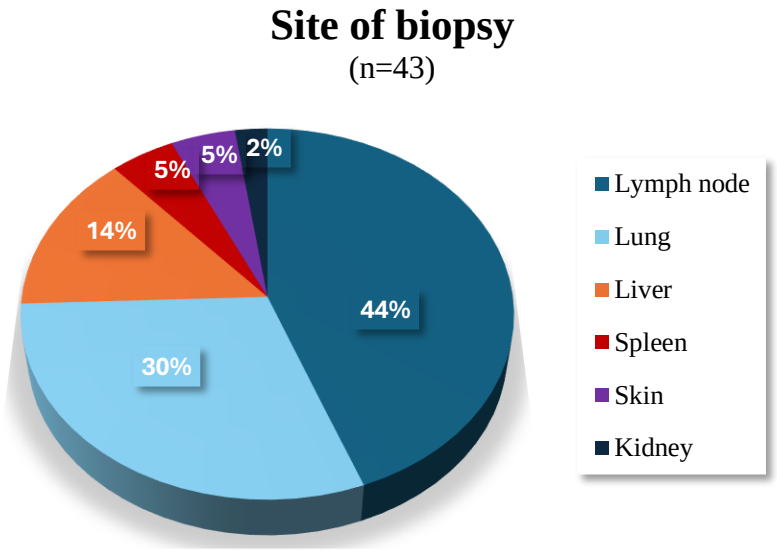

Figure 18: Distribution of sites biopsied for each patient. When a patient performed more than one biopsy in the same anatomic system (for example two biopsies in two lymph nodes), it was considered as one.

For each biopsy, we searched for three main characteristics:

- Fibrosis;
- Follicular lymphoid hyperplasia;
- Granulomas.

Lung biopsies and lymph node biopsies were analyzed separately, as higher numbers of exams were performed. The other biopsies were all collected together, as they had smaller numbers. The results are summarized in the following *Figures 19, 20 and 21*.

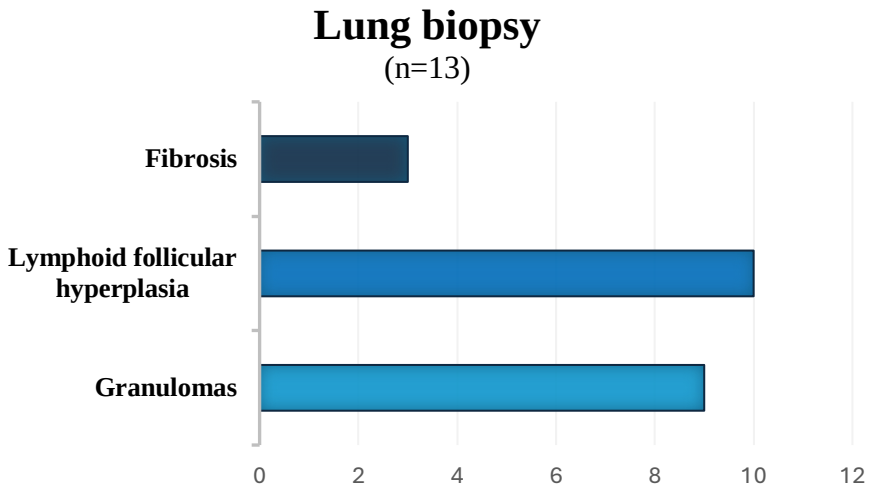

Figure 19: Main findings from lung biopsies.

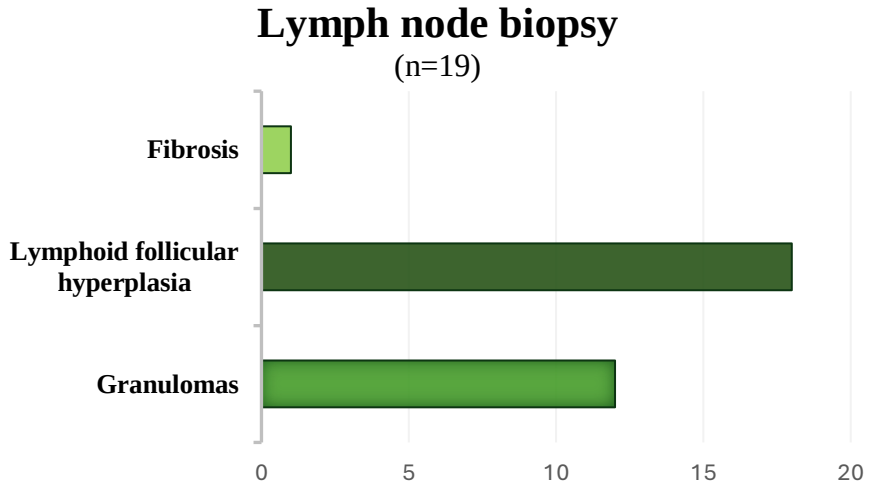

Figure 20: Main findings from lymph node biopsies.

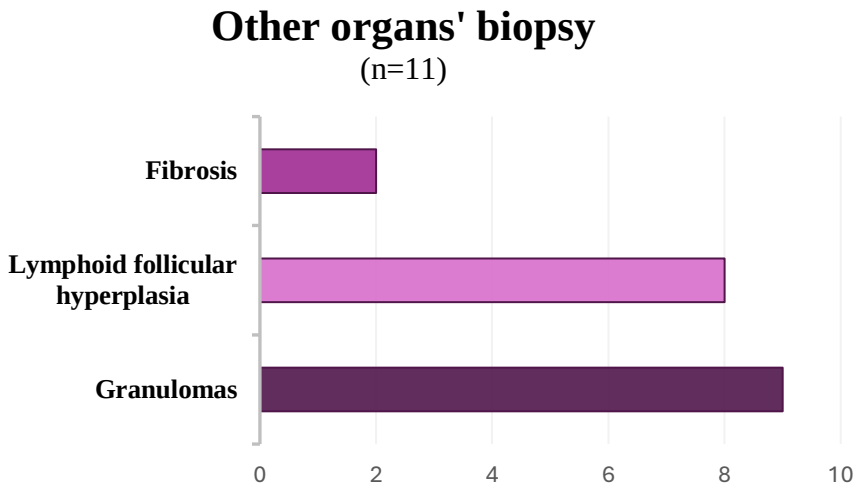

Figure 21: Main findings from other organs' biopsies.

5.1.3 Laboratory parameters

5.1.3.1 Immunoglobulin serum levels and Ig replacement therapy

Firstly, the immunoglobulin serum levels (IgG, IgA, IgM) at diagnosis of CVID were collected. Data were available for 57 patients, showing a median of 236.0 mg/dL for IgG (128.0-375.0), 10.0 mg/dL for IgA (5.5-24.0), and 18.0 mg/dL for IgM (9.0-44.3).

Then, we focused on the administration of immunoglobulin replacement therapy (IgRT). All 64 patients were under IgRT, and 50 of them got antibiotic prophylaxis (78%). The main route for administration of IgRT was subcutaneous (51 patients, 80%).

The data we searched for were:

- First and last available dose of IgRT;
- First and last available Ig serum level under IgRT, also defined as Ig trough level (IgTL).

The median of IgRT dose was 376.0 mg/kg/4 weeks (325.0-430.0) at first and 401.0 mg/kg/4 weeks (353.0-474.0) at last available measurement, with a significant increase (*p=0.002***). IgTL median was 597.0 mg/dL (412.0-743.0)

at the first available measurement and 738.0 mg/dL (628.0-877.0) at the last, again with a significant variation ($p<0.001$***).

5.1.3.2 Analysis of serum B and T cell subpopulations

We analyzed B and T cell subpopulations from the serum of 56 patients, based on data availability.

In particular, the T cells percentage (CD3+%) was calculated, along with the percentages of T helper cells (CD4+%), T cytotoxic cells (CD8+%), and their ratio (CD4+/CD8+).

Then, we considered the B cell percentage (CD19+%) and we collected data regarding the B-cell immunophenotyping according to the EUROclass protocol.

B and T subsets of our cohort are presented in *Table III*.

Table III: B and T cell subpopulations

Median and interquartile of B and T cell subpopulations. Naïve B cells = CD27-IgM+IgD+; marginal zone B cells = CD27+IgM+IgD+; switched memory B cells = CD27+IgM-IgD-; transitional B cells = CD38++IgM++; plasmablasts = CD38+++IgM-; activated B cells = CD21lowCD38low.

CD3+%	79.00 (72.00-84.00)
CD4+%	46.00 (39.00-51.30)
CD8+%	28.50 (21.90-37.50)
Ratio CD4+/CD8+	1.27 (0.85-1.82)
CD19+%	8.00 (5.85-12.00)
Naïve %	81.2 (70.0-88.3)
Marginal zone %	4.50 (2.00-14.80)
Switched memory %	1.80 (0.85-2.67)
Transitional %	1.90 (0.95-4.05)
Plasmablasts %	0.90 (0.20-1.47)
CD21^{low} %	13.20 (4.65-30.30)
CD3+CD57+ %	14.00 (6.10-21.50)

5.1.3.3 Analysis of the bronchoalveolar lavage fluid (BALF)

A total of 36/64 patients performed an analysis of the differential cell count and lymphocyte phenotyping on bronchoalveolar lavage fluid (BALF).

The median cell count was 155.00/mm^3 (81.00-279.00). The other results are expressed in the following *Table IV*.

Table IV: BALF features

Median and interquartiles of BALF features.

Macrophages %	41.00 (11.50-66.30)
Neutrophils %	12.00 (2.75-39.50)
Eosinophils %	0.00 (0.00-0.50)
Lymphocytes %	25.00 (13.50-50.00)
CD3+%	89.70 (85.80-95.00)
CD4+/CD8+ ratio	1.45 (1.02-2.58)
CD19+%	2.92 (1.15-7.84)
Switched memory %	11.80 (2.83-13.30)
Naïve %	13.30 (2.83-22.50)
Marginal zone %	1.85 (0.40-3.15)
CD21^{low} %	75.50 (61.50-80.80)
Transitional %	0.00 (0.00-0.00)

5.1.4 Pulmonary function test (PFT)

We collected every pulmonary function test (PFT) that each patient performed, comparing them the first and the last available PFT.

The parameters we considered were:

- Forced expiratory volume in 1 second (FEV1);
- Forced vital capacity (FVC);
- Total lung capacity (TLC);
- Diffusing capacity of the lung for carbon monoxide (DLCO).

The analysis of the populations gave the results listed in the *Table V* below.

Table V: Lung function parameters of the whole cohort.

Median and interquartile of FEV1, FVC, TLC, and DLCO and percentage of predicted of the study population at first and last available PFT

First PFT	
FEV1 (L)	2.65 (2.16-3.29)
FEV1%	86.00 (75.00-99.00)
FVC (L)	3.37 (2.64-4.11)
FVC%	87.00 (74.00-99.00)
TLC (L)	4.91 (2.96-5.44)
TLC%	85.00 (79.00-96.30)
DLCO (mL/min/mmHg)	18.5 (14.7-22.9)
DLCO%	77.00 (66.00-91.00)

Last PFT	
FEV1 (L)	2.58 (2.08-3.05)
FEV1%	86.0 (71.80-93.80)
FVC (L)	3.26 (2.80-3.87)
FVC%	87.50 (74.30-102.00)
TLC (L)	4.79 (4.19-5.34)
TLC%	85.00 (74.80-100.00)
DLCO (ml/min/mmHg)	17.1 (11.8-20.1)
DLCO%	75.00 (59.00-89.00)

No statistically significant variation was found in the evaluated parameters between the first and last PFT. However, for treated patients, the last PFT were performed after treatment, thus possibly masquerading the pre-treatment decline. We focused then on DLCO, observing that its values were interestingly lower than the general population. The difference can be seen in the following *Figure 22*.

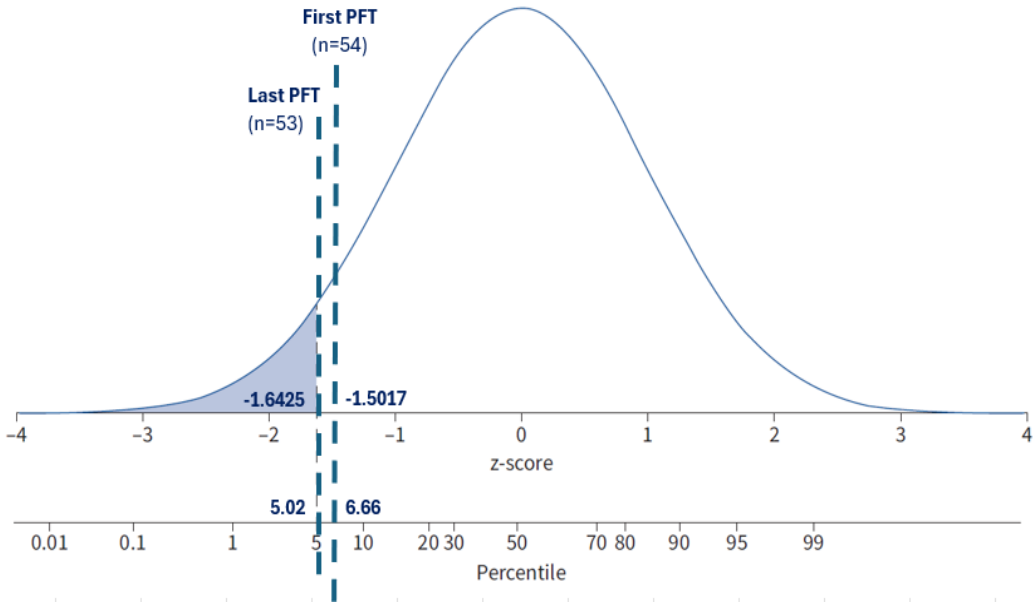

Figure 22: DLCO distribution at first and last available PFT, in comparison to the normal DLCO distribution. The graph was modified from the article "ERS/ATS technical standard on interpretive strategies for routine lung function tests[1023].

5.1.5 Genetics

Genetic testing results were available for 22 patients: out of them, 10 were negative. Focusing on the 12 positive tests, we obtained 13 different mutations, as some patients had more than one. The most common finding was the mutation of TACI, the BAFF receptor already mentioned in *Paragraph 2.5.3*. TACI is part of the TNF receptors superfamily, and interestingly we noticed that also other two TNF receptors were obtained from the genetic tests: TNFRSF12 and TNFRSF1A. Another common feature was the mutation of CTLA4, also already mentioned in *Paragraph 2.5.3*. The results are summarized in *Figure 23*.

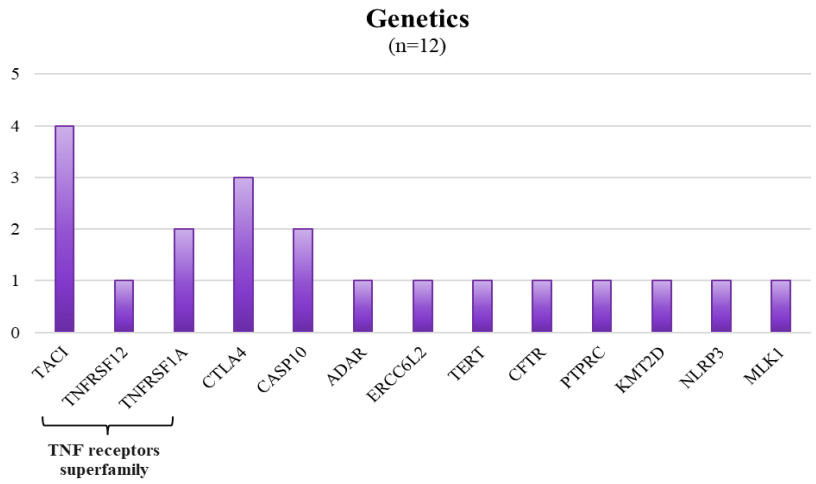

Figure 23: Genetic test results

5.1.6 Clinical manifestations

Based on current knowledge, GLILD seems to be a lung manifestation of a systemic disease rather than a standalone lung condition. This was evident in our sample, as illustrated in *Figure 24*, where 85% of the participants showed extrapulmonary involvement.

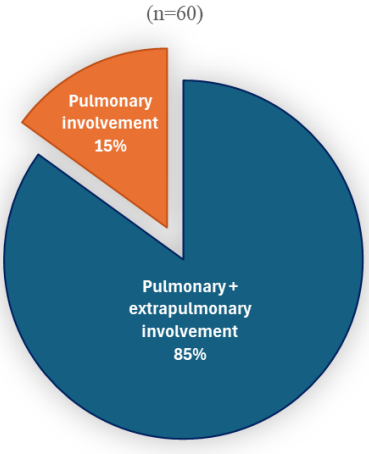

Figure 24: GLILD's organ involvement

In particular, spleen involvement was very common (*see Figure 25*), as 77% of our patients presented splenomegaly (defined as diameter >13 cm). Of this subgroup, 8 subjects (13% of the total) underwent splenectomy.

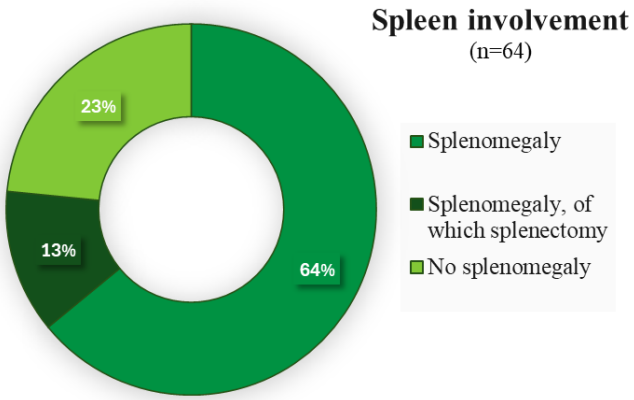

Figure 25: Spleen involvement in our population.

We then classified the patients using the Chapel classification described in Paragraph 2.5.5.1. As expected, no patients fell into the first category ("infection only"). As shown in *Figure 26*, the most prevalent category was "polyclonal lymphoproliferation" (III), as GLILD typically involves lymphoproliferation among its characteristics, followed by the "cytopenias" (II) category.

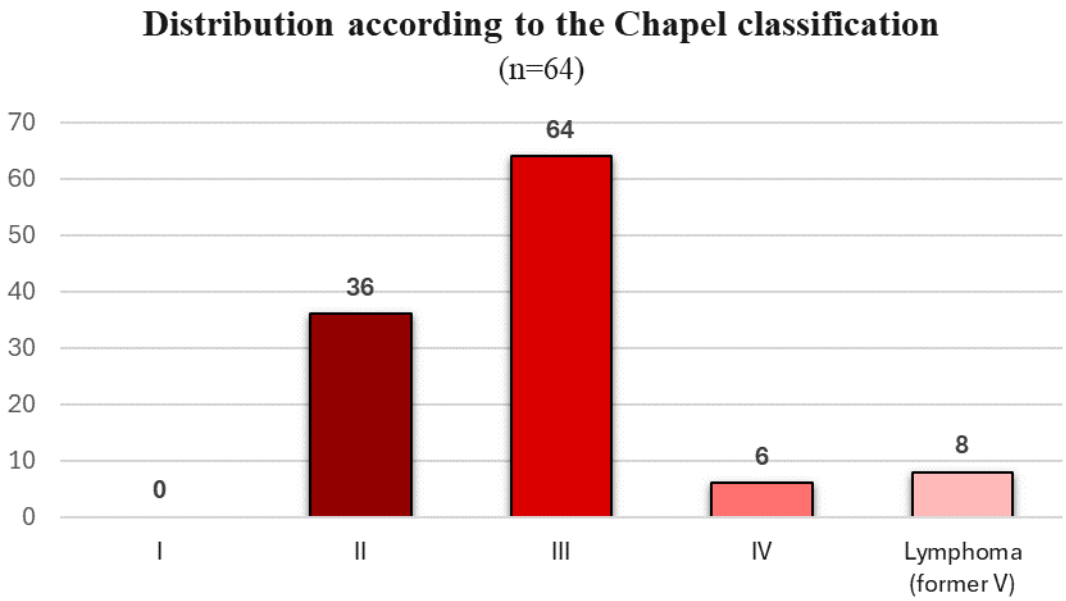

Figure 26: Chapel classification of the population: I: "infection only phenotype"; II: cytopenia; III: polyclonal lymphoproliferation; IV: enteropathy; V: former lymphoma.

Focusing on autoimmunity, we listed all the manifestations, and we collected them under groups, according to the organ involvement or the systemic dissemination.

For what concerns organ autoimmunity (*Figure 27*), we also specified if some diseases were more common than others, but we generally preferred to enlighten the organ involved, rather than the singular disease. Hepatopathy was predominant in the cohort (representing 31% of the total).

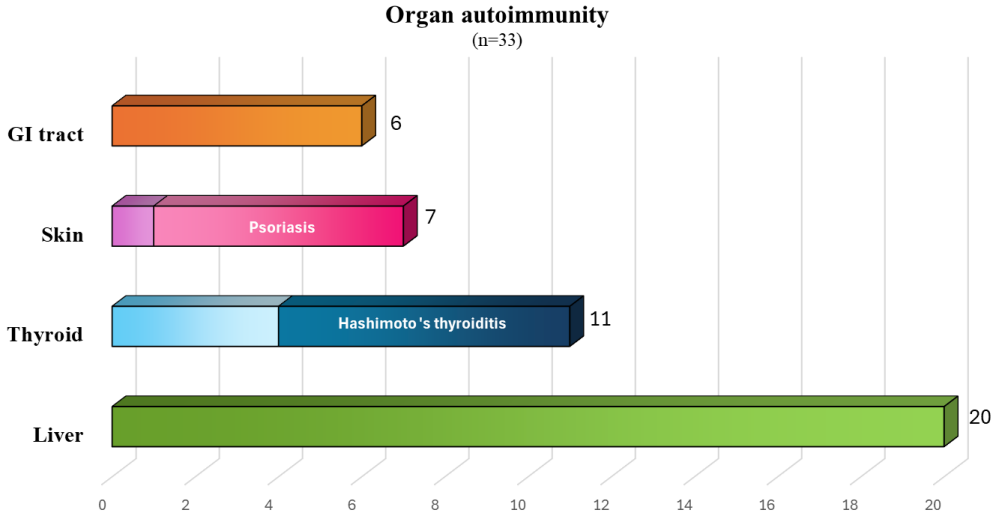

Figure 27: Organ autoimmunity in GLILD, divided by type of organ. Most common diseases are emphasized.

Afterward, we considered systemic involvement. It appeared to be quite common, with the leading disease being immune thrombocytopenia (ITP), followed by autoimmune hemolytic anemia (AIHA), as summarized in *Figure 28*.

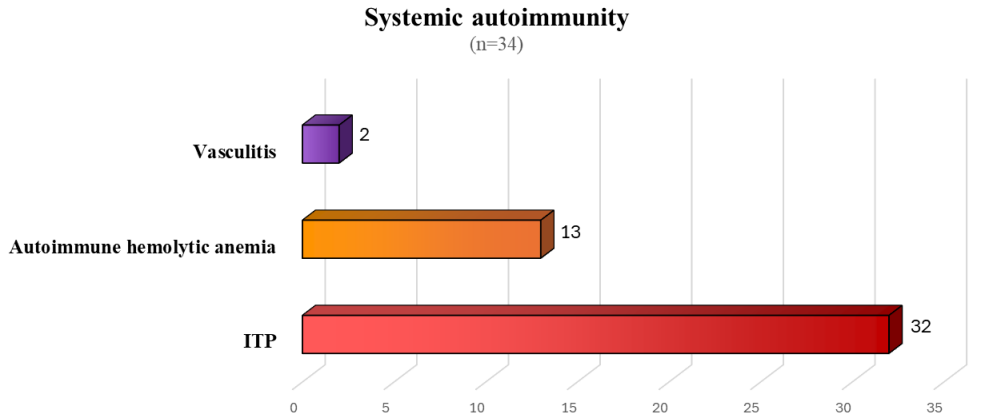

Figure 28: Main manifestations of systemic autoimmunity in GLILD.

Finally, we observed other complications, such as malignancies. Out of 64 patients, 16 had history of cancer, half of which had solid cancer and half hematologic (*see Figure 29*), most of which were lymphomas.

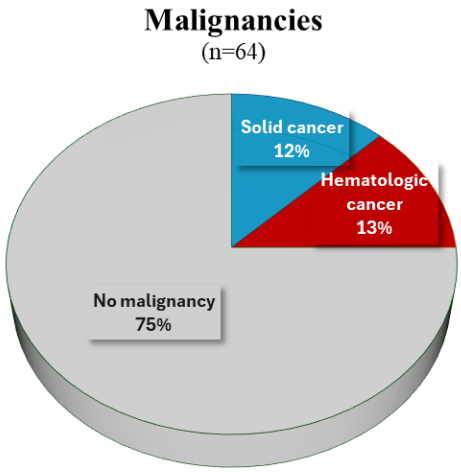

Figure 29: Prevalence of malignancies in the whole population.

5.1.7 CT scan and ^{18}FDG-PET-CT

We analysed the main findings of CT and ^{18}FDG-PET-CT of the whole population through the years. We analysed the main findings of CT scan and ^{18}FDG-PET-CT of the whole population through the years. CTs from 52 patients and ^{18}FDG-PET-CTs from 33 patients were collected.

For CTs, we gained information about four characteristics:

- Lymphadenopathies;
- Bronchiectasis;
- Nodules;
- Ground glass opacities (GGOs).

For ^{18}FDG-PET-CTs, we investigated if there was an involvement of:

- Liver;
- Spleen;

- Lung;
- Subdiaphragmatic lymph nodes;
- Supradiaphragmatic lymph nodes.

The results are showed in the *Figures 30 and 31* below.

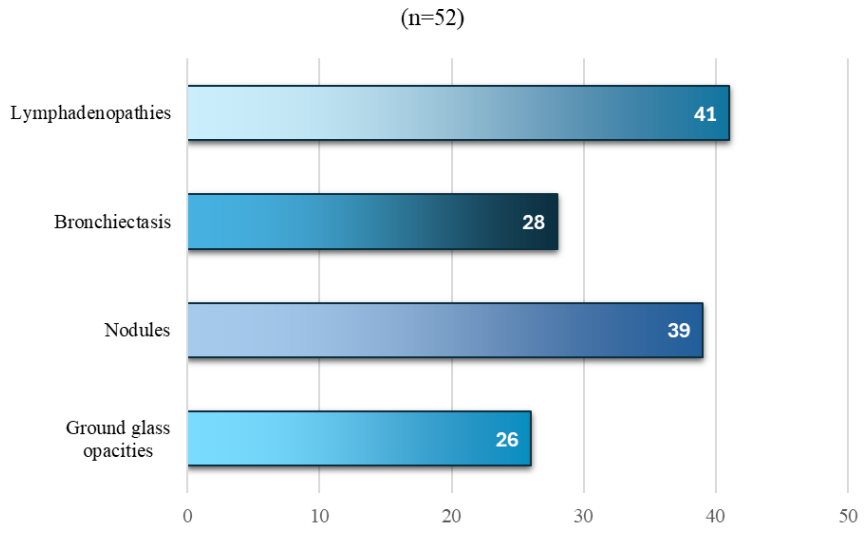

Figure 30: Findings from first available chest CT scan

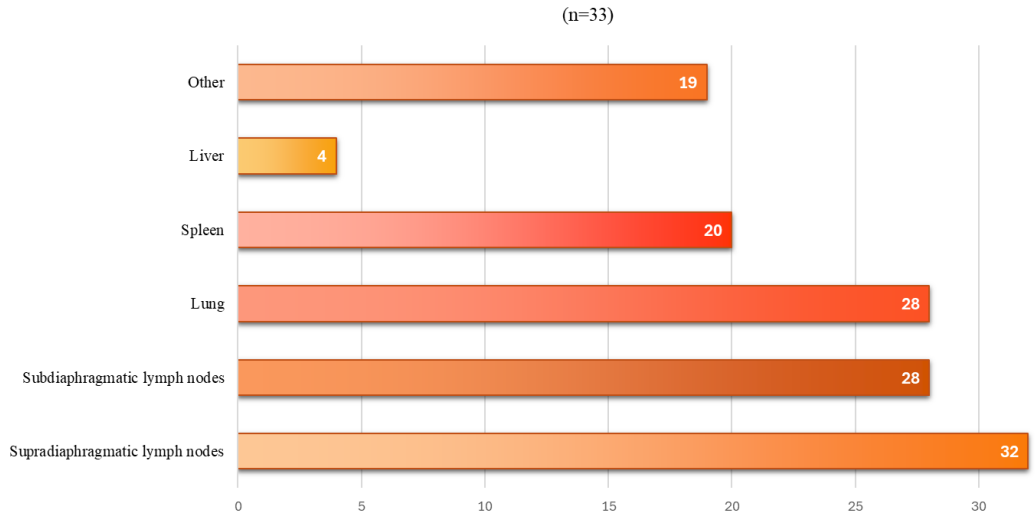

Figure 31: Organ involvement from first available ^{18}FDG-PET-CT

5.1.8 Mortality

Out of our 64 patients, 11 died, representing the 17% of the sample. The Kaplan-Meier curve we obtained is presented in the *Figure 32* below.

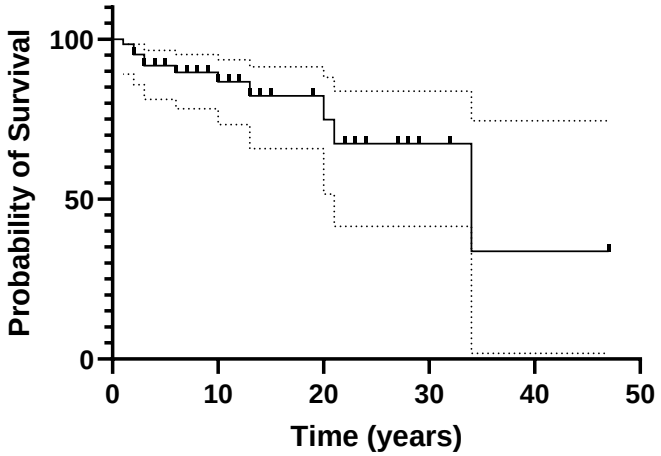

Figure 32: Kaplan-Meier curve of the sample. The independent variable is represented by the years between the diagnosis of CVID and the death or the last follow-up of the patient, while the independent variable is the probability of survival.

The causes of death are summarised in the following *Figure 36*. When death occurred due to infectious causes, these were of a pulmonary nature in patients with impaired lung function due to GLILD. When death was related to hepatic complications, it occurred in patients with hepatic involvement. In one case, renal failure was recorded as the cause of death in a patient with multi-organ damage related to complications of granulomatous disease

The causes of death are summarised in the following *Figure 33*.

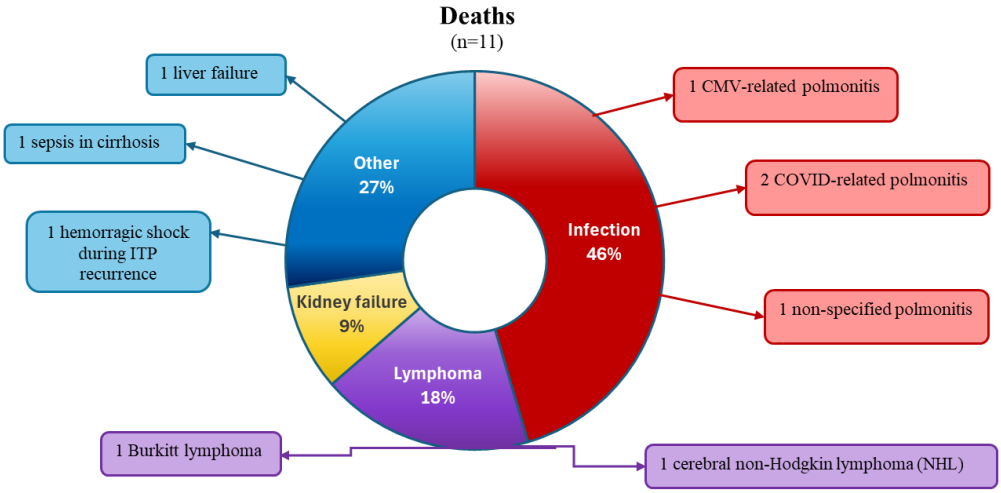

Figure 33: Causes of deaths, divided in subgroups (infection, lymphoma, kidney failure, other).

5.1.9 Treatment

All 64 patients were under Ig replacement therapy, as aforementioned; additionally, 40 of them received a specific GLILD therapy.

The different treatment strategies were represented by:

- Steroids;
- DMARDs (mainly Azathioprine and Mycophenolate);
- Rituximab (anti-CD20), either alone or in combination with DMARDs;
- Treatments for the complications, such as R-CHOP for lymphomas.

The data are shown in *Figure 34* below.

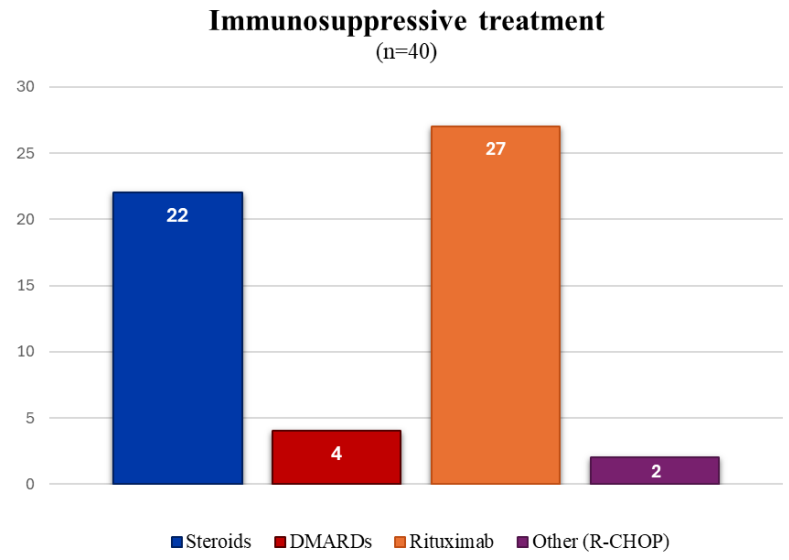

Figure 34: Treatment strategies used for our population.

The main reasons behind the treatment initiation were several: decline of lung function parameters (reduction of DLCO >10% or FVC >5%) or respiratory failure, radiological progression, extrapulmonary progression, and thrombocytopenia.

Steroids were administered at a median dose of 0.5 mg/kg, usually for cycles. Rituximab was administered mainly at a dosage of 375 mg/m^2/week for 4 weeks,

from 2 to 6 cycles. 14 patients who underwent Rituximab had clear available evidence of response (*e.g.* from CT, ^{18}FDG-PET-CT, PFT, or regression of symptoms).

5.2 Comparisons between case and control group

5.2.1 Demographic characteristics

The distribution of sex achieved statistical significance when comparing the case and control groups (*see Figure 35*).

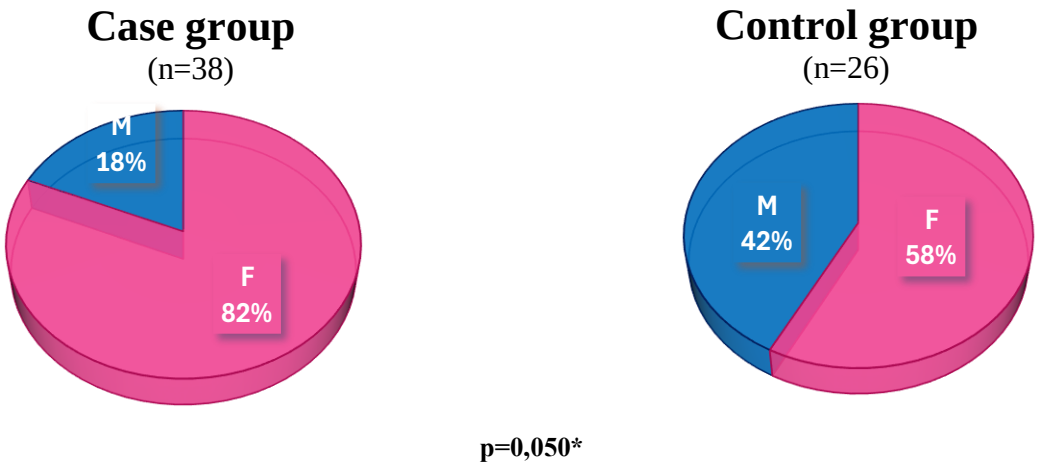

Figure 35: Sex distribution among case ("negative prognosis group") and control group ("positive prognosis group"). Using the Fisher's exact test, the analysis reached the upper limit of statistical significance (p=0,050)*

In the following *Table VI,* the demographic characteristics of the two groups are presented.

Table VI: Demographic characteristics of the sample

Median and interquartile range of age, age at diagnosis, age at first symptoms and diagnostic delay of respectively CVID and GLILD in the sample, divided between cases and controls. U Mann-Whitney test was performed to determine the p-value.

	Cases	Controls	P
Age	52.50 (43.00-67.25)	43.50 (36.50-53.00)	**0.029***
Age at first symptoms of IDCV	28.00 (13.75-36.00)	31.00 (21.25-38.25)	0.432
Age at first symptoms of GLILD	39.50 (28.50-53.00)	34.00 (31.00-47.00)	0.145
Age at diagnosis of CVID	39.00 (30.00-53.00)	35.00 (25.00-46.00)	0.222
Age at diagnosis of GLILD	47.00 (36.75-58.00)	39.00 (34.00-47.50)	0.109
Diagnostic delay of CVID	9.00 (4.00-16.00)	3.00 (0.00-6.00)	**0.002****
Diagnostic delay of GLILD	1.00 (0.00-7.00)	2.00 (0.00-8.00)	0.780

The only variables that presented a statistical significance were age (*p=0.029**) and diagnostic delay for the diagnosis of CVID (*p=0.002***).

5.2.2 Biopsies and histological findings

Comparisons between cases and controls did not lead to significant differences. The results are presented in the following *Table VII*.

Table VII: Histological findings

Frequencies and percentages (referred to as the total of the case/control group) of most common histological findings from respectively lung biopsy, lymph node biopsy, and other organs' biopsy, divided between cases and controls. Fisher's exact test was performed to determine the p-value. L.F.H. = lymphoid follicular hyperplasia.

	Cases	Controls	P
Granuloma – Lung	8 (80.0%)	1 (20.0%)	0.089
L.F.H. – Lung	7 (77.8%)	3 (60%)	0.580
Fibrosis – Lung	3 (33.3%)	0 (0.0%)	0.229
Granuloma – Lymph node	7 (70.0%)	5 (62.5%)	1.000
L.F.H. – Lymph node	12 (92.3%)	6 (75.0%)	0.531
Fibrosis – Lymph node	1 (7.7%)	0 (0.0%)	1.000
Granuloma – Other	5 (83.3%)	4 (50.0%)	0.301
L.F.H. – Other	3 (60.0%)	5 (100.0%)	0.144
Fibrosis – Other	1 (16.7%)	1 (12.5%)	1.000

Every finding was more common in the case group if considering lung and lymph node biopsies. Notably, fibrosis was absent in the control group in both types of biopsies. In any case, no statistical significance was reached.

5.2.3 Biohumoral exams

5.2.3.1 Immunoglobulin serum levels and Ig replacement therapy

The immunoglobulin serum levels (IgG, IgA, IgM) at diagnosis of cases and controls are summarized in the following *Table VIII*.

Table VIII: Immunoglobulin serum levels at diagnosis

Median and interquartile range of IgG, IgA, and IgM, divided between case and control groups. U Mann-Whitney test was performed to determine the p-value.

	Cases	Controls	p
IgG (mg/dL)	189.00 (79.00-320.00)	298.00 (189.50-397.80)	**0.027***
IgA (mg/dL)	8.00 (6.00-23.00)	16.50 (4.25-25.80)	0.673
IgM (mg/dL)	17.00 (8.00-56.00)	19.00 (16.00-35.50)	0.914

All the immunoglobulin levels were reduced in the case group, but the analysis reached statistical significance only for the IgG subgroup (*p=0.027**), as can be seen in *Figure 36*.

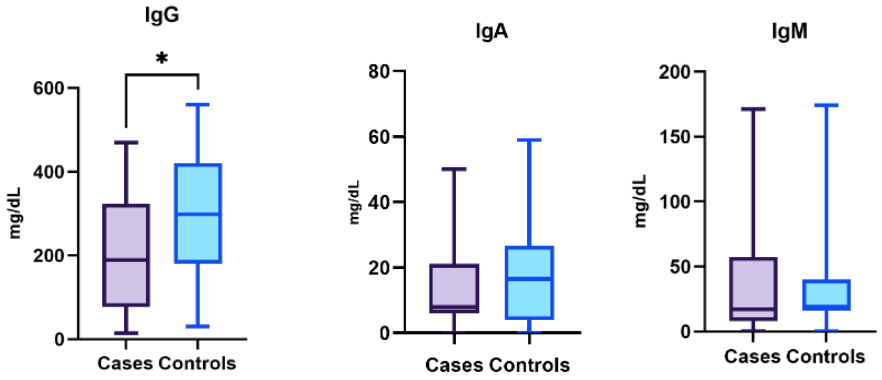

Figure 36: Comparison in IgG, IgA, and IgM serum levels at diagnosis between cases and controls.

Then, IgTL levels were analyzed. The data were collected at the first and last available IgRT dosage (steady state). The results are summarised in the following *Table IX*.

Table IX: First and last available IgRT doses and IgTL

The median and interquartile range of respectively first and last available IgRT and IgTL, divided between case and control groups. U Mann-Whitney test was performed to determine the p-value.

	Cases	Controls	p
First available IgRT (mg/kg/4 weeks)	400 (340-440)	357 (303-408)	0.169
First available IgTL (mg/dL)	581 (431-710)	629 (406-769)	0.653
Last available IgRT (mg/kg/4 weeks)	421 (377-495)	377 (332-437)	**0.042***
The last available IgTL (mg/dL)	762 (600-883)	726 (660-860)	0.797

The case group required the highest doses of IgRT and presented the lowest IgTL both at first and last observation. The levels of the last available IgRT were significantly different (*p=0.042**), as shown in *Figure 37*.

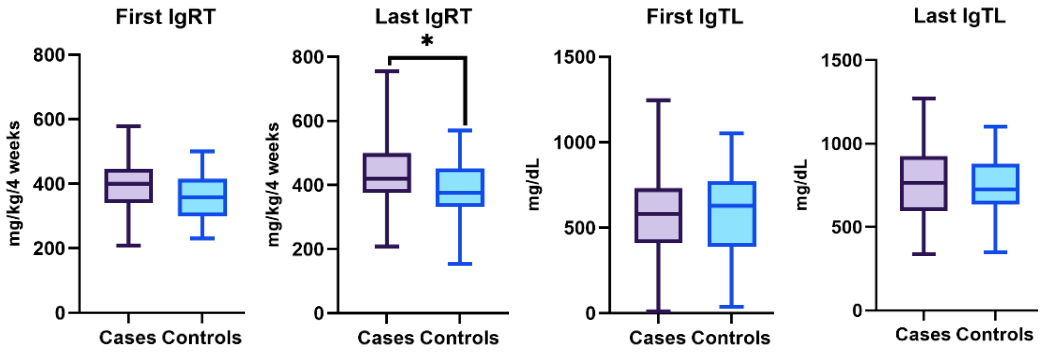

Figure 37: Comparison in IgRT doses and IgTL serum levels at diagnosis and last available control (steady state) between cases and controls.

5.2.3.2 Analysis of serum B and T cell subpopulations

Peripheral blood leukocytes median count was 4720 (3450-6500) for the case group and 5235 (3875-5800) for the control group (*p=0.951*). The median percentage of lymphocytes was 28% (22%-35.5%) for the cases and 33.55% (29.00%-38.25%) for the controls (*p=0.084*).

The results for T subgroups can be seen in the following *Table X*.

Table X: T cell percentage and subpopulations

Median percentage and interquartile of T cell subpopulations and median and interquartile of CD4+/CD8+ ratio, divided between cases and controls. U Mann-Whitney test was performed to determine the p-value.

	Cases	Controls	p
CD3+%	82.50 (76.75-85.00)	73.84 (71.00-79.00)	**0.010****
CD4+%	44.00 (38.50-51.25)	47.05 (40.57-51.50)	0.483
CD8+%	33.30 (22.75-42.50)	26.00 (21.30-33.50)	0.098
CD4+/CD8+ ratio	1.10 (0.80-1.48)	1.58 (1.20-2.33)	**0.050***

The total percentage of T cells (*p=0.010***) and the ratio CD4+/CD8+ (*p=0.050**) reached statistical significance, with cases having a lower CD4+/CD8+ ratio and a higher percentage of CD3+.

The results of the B-cell immunophenotyping according to the EUROclass protocol are summarised in *Table XI* below.

Table XI: B cells percentage and subpopulations

Median percentage and interquartile of B cells and subpopulations, divided between cases and controls. U Mann-Whitney test was performed to determine the p-value.

	Cases	Controls	p
CD19+%	7.60 (4.40-10.00)	10.00 (6.09-15.00)	**0.045***
Naïve %	80.60 (69.38-89.17)	84.00 (70.00-88.10)	0.948
Marginal zone %	6.40 (2.50-14.55)	3.60 (1.80-12.85)	0.586
Switched memory %	1.80 (1.08-2.20)	2.15 (0.85-3.28)	0.741
Transitional %	1.55 (0.60-4.85)	2.30 (1.45-3.90)	0.712
Plasmablasts %	0.80 (0.17-1.30)	1.00 (0.30-1.70)	0.469
CD21^{low} %	11.55 (4.65-27.25)	17.75 (4.70-37.33)	0.485
CD3+CD57+%	12.60 (9.00-17.50)	14.50 (5.90-30.0)	0.662

The only significant difference we found is the total percentage of B cells *(p=0.045*),* with cases showing a lower percentage of CD19+.

5.2.3.3 Analysis of the bronchoalveolar lavage fluid (BALF)

The median cell count was 145.00/mm^3 (70.00-239.00) for cases and 177.00/mm^3 (87.00-400.00) for controls (*p=0.583*).

Percentages of the main cell populations gave these results:

- Macrophages: 32.00% (7.00-61.500) for cases, 52.50% (36.25-69.50) for controls (*p=0.328*);
- Neutrophils: 33.50% (9.25-51.50) for cases, 3.00% (0.25-9.50) for controls (*p=0.026*);*
- Eosinophils: 0.00% (0.00-1.00) for cases and 0.00% (0.00-0.00) for controls (*p=0.449*);
- Lymphocytes: 20.50% (12.60-34.75) for cases and 30.00% (22.50-58.50) for controls (*p=0.247*).

For what it concerns lymphocyte subgroups, the available data are summarized in the following *Table XII.*

Table XII: BALF features

Median and interquartiles of BALF features in cases and controls. U Mann-Whitney test was performed to determine the p-value.

	Cases	Controls	p
CD3+%	88.00 (82.00-93.50)	94.50 (90.63-95.75)	0.083
CD4+/CD8+ ratio	1.10 (0.520-1.45)	4.13 (2.16-6.09)	**0.004****
CD19%	4.80 (1.50-8.22)	1.60 (1.20-2.00)	0.315
Switched memory %	11.30 (5.65-11.80)	13.70 (6.85-23.95)	0.507
Naïve %	11.30 (5.65-20.15)	15.30 (7.65-20.10)	1.000
Marginal zone %	2.10 (1.05-2.80)	1.60 (0.80-4.30)	1.000
CD21^{low} %	78.00 (70.5-81.50)	59.40 (51.45-70.20)	0.517
Transitional %	0.00 (0.00-0.00)	0.00 (0.00-0.10)	0.505

Only the ratio CD4+/CD8+ reached the statistical significance (*p=0.004***); interestingly, this parameter was significant also for the serum levels analysis, as seen in *Paragraph 5.2.3.2*.

5.2.4 Pulmonary function test (PFT)

The results of the comparison between the case and control group at the first and last available PFT are summarised in *Tables XIII and XIV* below.

Table XIII: Lung function parameters at first available PFR

FEV1, FVC, TLC, DLCO, and percentages of predicted at first available PFR, divided between case and control group. U Mann-Whitney test was performed to determine the p-value.

	Cases	Controls	p
FEV1 (L)	2.33 (1.98-3.04)	3.08 (2.55-3.59)	**0.004****
FEV1 %	82.00 (70.50-96.50)	91.50 (77.50-105.00)	0.206
FVC (L)	2.95 (2.34-3.74)	3.94 (3.31-4.62)	**<0.001*****
FVC %	82.00 (72.50-95.00)	97.00 (89.00-103.00)	**0.013***
TLC (L)	4.20 (3.77-5.47)	5.29 (5.21-5.39)	0.144
TLC %	80.00 (72.50-93.50)	95.00 (85.00-101.00)	**0.028***
DLCO (mL/min/mmHg)	16.20 (12.20-19.40)	22.90 (18.90-25.10)	**<0.001*****
DLCO %	70.50 (58.00-80.80)	91.00 (80.00-100.00)	**0.002****

Table XIV: Lung function parameters at last available PFR

FEV1, FVC, TLC, DLCO, and percentages of predicted at last available PFR, divided between case and control group. U Mann-Whitney test was performed to determine the p-value.

	Cases	Controls	p
FEV1 (L)	2.23 (1.74-2.87)	2.75 (2.46-3.39)	**0.006****
FEV1 %	83.00 (71.00-90.00)	88.00 (78.00-101.00)	0.127
FVC (L)	2.87 (2.43-3.68)	3.76 (3.25-4.44)	**<0.001*****
FVC %	79.00 (70.00-96.00)	100.00 (83.00-103.00)	0.044
TLC (L)	4.50 (3.76-4.92)	5.24 (4.50-5.56)	0.063
TLC %	83.00 (74.00-94.00)	93.00 (81.50-102.00)	0.293
DLCO (mL/min/mmHg)	16.3 (11.1-18.4)	19.2 (15.9-27.0)	0.072
DLCO %	72.50 (58.30-86.50)	82.00 (73.00-90.00)	0.330

In *every* parameter considered, the case group had lower values than the control group. Moreover, the analysis reached a high level of significance for what it concerns FEV1 and FVC at both PFRs and DLCO at the last PFR (respectively *p=0.004*** and *p=0.006*** for FEV1 at first and last PFR, *p<0.001**** for FVC in both first and last PFR, *p<0.001**** for DLCO at first PFR).

Additionally, we analyzed the DLCO values in both cases and controls, as this parameter showed significant differences in both absolute numbers and percentage of predicted values. Notably, the DLCO in the case group was close to the 5th percentile of the normal distribution at both the first and last available pulmonary function tests (PFTs). In contrast, the DLCO values in the control group remained much higher, around the 40th to 50th percentiles. *Figures 37* and *38* visually illustrate this comparison.

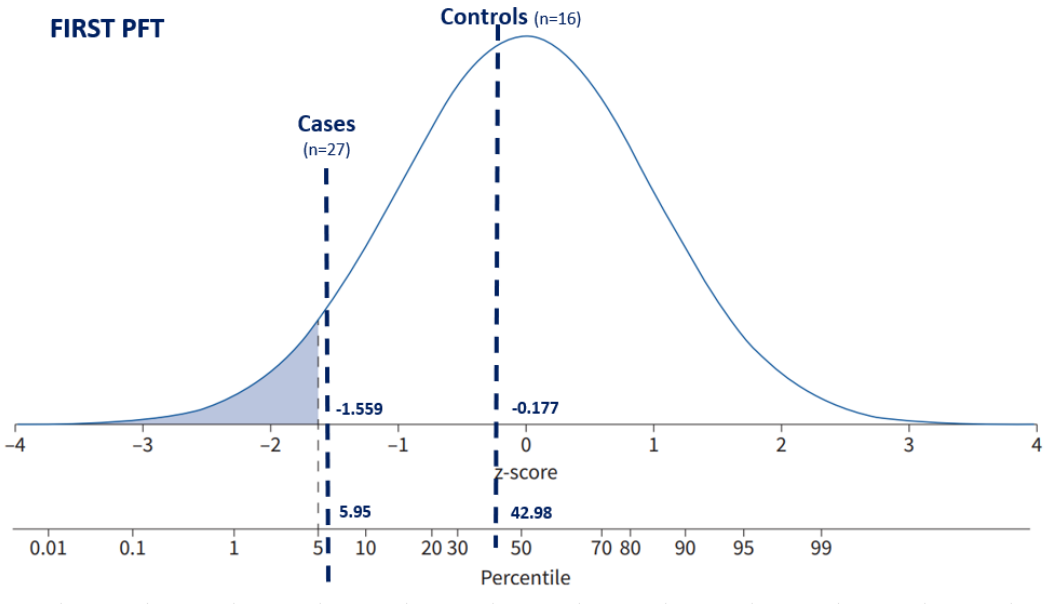

Figure 37: DLCO distribution of case and control group at first available PFT, in comparison to the normal DLCO distribution. The graph was modified from the article "ERS/ATS technical standard on interpretive strategies for routine lung function tests"[102].

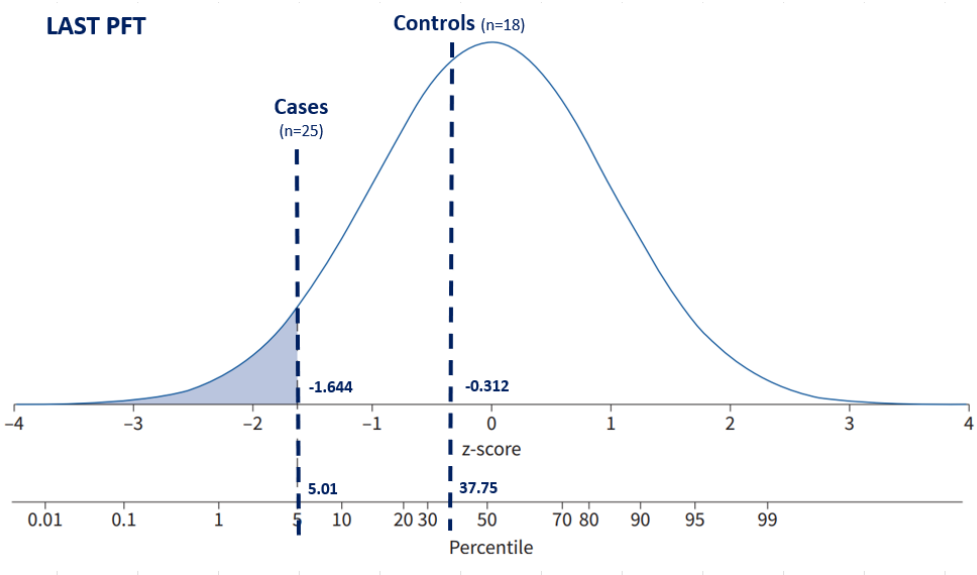

Figure 38: DLCO distribution of case and control group at last available PFT, in comparison to the normal DLCO distribution. The graph was modified from the article "ERS/ATS technical standard on interpretive strategies for routine lung function tests"[102].

5.2.5 Clinical manifestations

The analysis of clinical manifestations yielded the following results:

- There was no significant difference between cases and controls regarding splenomegaly (p=0.630) or splenectomy (p=0.364).
- According to the Chapel classification, no significant association was found between the two groups. Chapel I and II were excluded from the comparison, as no patients were classified as Chapel I and all were classified as Chapel II. In Chapel III, 21 cases and 15 controls were included (p=0.896), while 3 cases and 3 controls were classified as Chapel IV (p=0.623).
- Autoimmune cytopenia was more common in the case group, but the difference was not statistically significant (p=1.000).
- Interestingly, organ autoimmunity was more frequent in the control group, though the difference did not reach statistical significance (p=0.175).
- No comparison was made between cases and controls regarding malignancies, as lymphomas were part of the inclusion criteria for the case group.

5.2.6 CT scan and ^{18}FDG-PET-CT

In the following *Table XV* are summarised the main findings at first available CTscan.

Table XV: Main findings at first available CT scan

Absolute number and percentage (referred to the total of respectively the case group or the control group) of CT findings at first available scan, divided between cases and controls. Fisher's exact test was performed to determine the p-value.

	Cases	Controls	p
GGO	22 (71.0%)	4 (19.0%)	**<0.001*** **
Nodules	26 (83.9%)	13 (61.9%)	0.105
Bronchiectasis	22 (71.0%)	6 (28.6%)	**0.004** **
Lymphadenopathies	27 (87.1%)	14 (66.7%)	0.095

The frequence of the parameters was *always* higher in the case group than in the control group. Statistical significance was reached considering ground glass opacities (*p<0.001****) and bronchiectasis (*p=0.004***).

The main ^{18}FDG-PET-CT findings are listed in the *Table XVI* below.

Table XVI: Main findings at first available ^{18}FDG-PET-CT

Absolute number and percentage (referred to the total of respectively the case group or the control group) of ^{18}FDG-PET-CT findings at first available scan, divided between cases and controls. Fisher's exact test was performed to determine the p-value.

	Cases	Controls	p
Supradiaphragmatic lymph nodes	22 (100.0%)	10 (90.9%)	0.333
Subdiaphragmatic lymph nodes	10 (90.9%)	18 (81.8%)	0.643
Lung	20 (90.9%)	8 (72.7%)	0.304
Spleen	12 (54.5%)	8 (72.7%)	0.456
Liver	3 (13.6%)	1 (9.1%)	1.000

The frequency of involvement of almost every organ is higher in the case group than the control group, except for spleen involvement (but we already stated that it was a very common feature in the whole group). The totality of the case group presented supradiaphragmatic lymph nodes involvement. Moreover, every patient had at least one extra thoracic site. Anyway, no parameter reached the statistical significance.

5.2.7 Mortality and treatment

Comparisons in mortality and immunosuppressive treatment were not analysed, as they were both inclusion criteria for the case group.

5.3 Possible prognostic markers

Further analyses were performed to enlighten if we could use some of the parameters that had a significant difference between cases and controls as prognostic markers.

Starting from the above-listed results, we considered three quantitative variables: diagnostic delay from diagnosis of CVID, IgG levels at diagnosis and DLCO% of predicted. A logistic regression analysis was performed for each variable.

The following *Figures 39, 40, 41* demonstrate the correlation between the variations of these parameters and the risk of developing a more severe condition.

The p-values for each analysis were respectively *p=0.059* for DLCO%, *p=0.050** for IgG levels, *p=0.024** for the diagnostic delay.

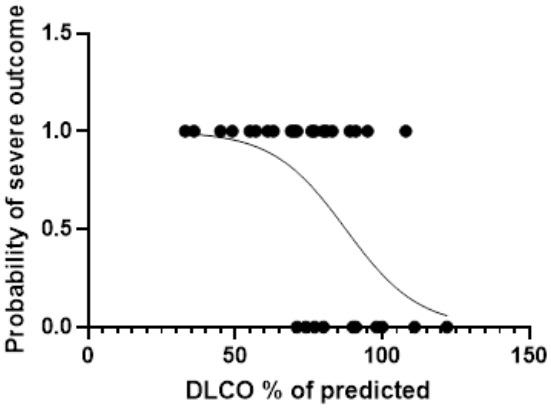

Figure 39: This graph shows that as DLCO% decreases, the probability of developing a more severe outcome increases.

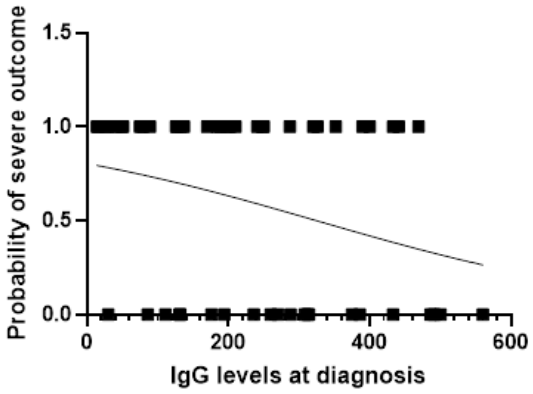

Figure 40: This graph shows that as IgG levels decreases, the probability of developing a more severe outcome increases.

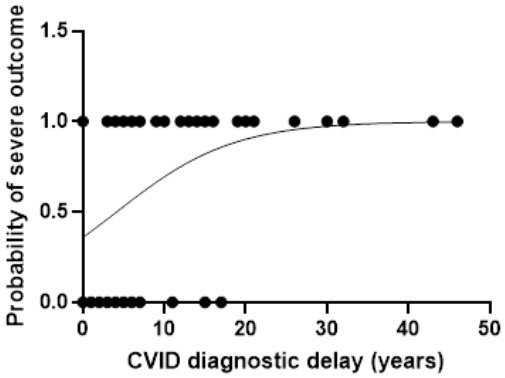

Figure 41: This graph shows that as the years of diagnostic delay of CVID rise, the probability of developing a more severe outcome increases.

The results of the multivariate analysis are shown in the *Table XVII* below.

Table XVII: results of the multivariate logistic regression analysis.

p-values and Odds Ratio (with percentiles) of the multivariate regression analysis we conducted on DLCO%, IgG levels ad diagnosis and years of CVID diagnostic delay.

	p	OR
DLCO% of predicted	0.090	1.007 (0.999-1.010)
IgG levels at diagnosis (mg/dL)	0.075	1.063 (0.991-1.140)
CVID diagnostic delay (years)	0.084	0.831 (0.674-1.020)

We calculated specificity, sensitivity and the ROC curve of a hypothetic prognostic score including these variables. We obtained a specificity of 0.952, a sensitivity of 0.750 and an Area under the curve (AUC) of 0.901, as presented in the *Figure 42* below.

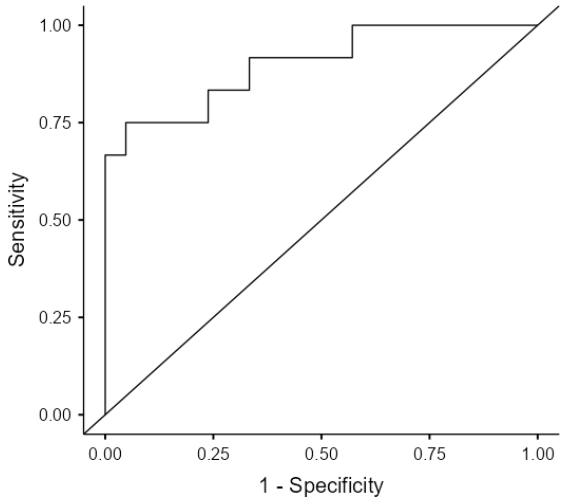

Figure 42: ROC curve of the results conducted on IgG serum levels, diagnostic delay, DLCO%.

6. Discussion

GLILD remains a poorly understood and relatively rare condition, classified as an interstitial lung disease in patients with CVID, characterized by lymphocytic infiltrates and/or granulomas in the lungs[25]. Currently, there is no established diagnostic algorithm or standardized treatment protocol for determining when to initiate therapy or which medications are most effective. Additionally, existing studies are limited to retrospective analyses with small patient cohorts, leaving many aspects of the disease unexplored.

Common variable immunodeficiency is the most prevalent symptomatic primary antibody deficiency (PAD) of the adult[4,7,26] and the most diagnosed symptomatic IEI. Rather than a single disease, its definition gathers together several disorders with or without a specific genetic background. As testified by the occurrence of GLILD, CVID is not just an "immune deficiency", but a condition where immune dysregulation may lead to infections or autoimmunity or both.[103]

The aim of our study was to analyse a multicenter cohort of GLILD patients from various perspectives, including clinical manifestations, mortality, radiological findings, laboratory parameters, and treatment strategies. By dividing patients into cases and controls, we sought to identify potential markers that could help distinguish between milder and more severe disease phenotypes.

Our findings suggest that GLILD predominantly affects females, which aligns with the general tendency of women to develop autoimmune diseases. However, this female predominance has not been clearly established in the existing literature[104,105].

Secondly, we observed that GLILD could appear before CVID diagnosis. This was already mentioned in literature[42], and in our sample 28% of the enrolled patients had symptoms of GLILD as the first clinical manifestation of CVID. This supports the importance of spreading the knowledge of this peculiar ILD, since it may lead to CVID diagnosis with obvious therapeutic implications.

Thirdly, as suspected, we noticed an important diagnostic delay[26]. Notably, our data show that the diagnostic delay for CVID remains longer than that for GLILD.

This may be because once CVID is diagnosed, patients typically undergo comprehensive screening, including pulmonary function tests and chest CT scans, to further assess their condition. Additionally, once CVID is identified, the likelihood of suspecting and diagnosing GLILD increases, leading to a shorter time to diagnosis for GLILD.

We also confirmed that the prognostic impact of GLILD is relevant since we found a mortality of 17% in our cohort, which is comparable to what has been reported in the last few years: a mortality rate of around 20%[41,83]. The causes of death were various, most of which strongly connected with GLIL D: in particular, fatal infections mainly involved the already damaged patients' lungs, while lymphomas are linked to the lymphoproliferative aspect of the disease; of note, the prevalence of lymphomas in our population was 12.5%, significantly higher than what reported in the Italian CVID population (5.5%)[106,107]. Lastly, hepatopathy and immune cytopenia[65,108,109] are well-known complications/co-morbidities of GLILD. Consequently, although GLILD is classified as an interstitial lung disease, our data clearly show that this is rather a lung manifestation of a wider, multisystemic condition, with a large spectrum of immune dysregulation, in line with what is reported in recent literature [83]. Indeed, we found at least one extrapulmonary involvement in 100% of cases.

Regarding extrapulmonary involvement, we then compared our data with the study by Jolles et al.[67] The findings in common were several, including:

- Splenomegaly;
- Widespread lymphadenopathy;
- Thrombocytopenia;
- Liver disease and/or enteropathy.

Splenomegaly was very common in our sample (77%), confirming its crucial role in the diagnostic process. Lymphadenopathy was the second most common manifestation, involving 72% of the population.

Systemic involvement was almost completely represented by autoimmune cytopenias, in particular immune thrombocytopenia (ITP), which affected half

the population. Autoimmune hemolytic anemia was present in 20% of the patients, being the second most frequent systemic comorbidity. Concentrating on single-organ comorbidities, hepatopathy (31%) was by far the most common in our population. Thyroiditis (17%), skin involvement (11%), and enteropathy (1%) were also diagnosed in some patients.

In terms of immunologic biomarkers, we also found many similarities with what was reported in other smaller cohorts (e.g. Switched memory B cells <2% and $CD21^{low}$ B cells >10%)[56,64,90].

Focusing on immunoglobulin levels, we observed a decrease in IgG, IgA, and IgM serum levels of our GLILD patients. On the contrary, we did not find a significant rise in IgM, as mentioned in the literature [58]. The whole cohort was receiving immunoglobulin replacement therapy, with satisfying results. Subcutaneous administration was the preferred route of administration, as it was used by 80% of our population; this preference was already supported by literature, as it appears to be safer and more efficient[18].

Laboratory tests gave some interesting results: the first and most important aspect is that $CD21^{low}$ B cell % was quite high in both analyses. This increase has been already discussed widely in literature, both for its potential diagnostic and prognostic implications[66,75]. Several studies suggested that these cells might have a key role in autoimmunity development, as they have already been found in rheumatoid arthritis and in Sjogren's syndrome[110]. $CD21^{low}$ B cells increase is associated with a significant decrease in SmB cells (mainly <2%) and by an increase in large granular lymphocytes, which has already been reported by our group[66].

BALF analysis of our patients presented a more than doubled percentage of lymphocytes. Among lymphocytes, CD4/CD8 ratio of T cells was on average normal, different from what is commonly found in another granulomatous ILD as sarcoidosis[71,76]. B cell subpopulations varied vastly, but the key finding was the clear predominance of activated $CD21^{low}$ B cells, representing more than 75% of total B cells; this is in line with other reports on small cohorts of GLILD patients[72] and strongly supports a pathogenetic role of this subpopulation, with

clear therapeutic implications. Putting together peripheral blood and BALF findings, these alterations seem to confirm the state of imbalance that characterizes GLILD patients' immune system [97].

Genetic tests of our GLILD patients confirmed the already mentioned and well-known roles of TACI[60] and CTLA-4[53] in the pathogenesis of the disease. Unfortunately, the sample size and the incomplete coverage of genetic screening in our cohort do not allow us to draw any conclusions.

Concerning GLILD diagnosis, PFTs, radiology, and biopsies' results are aligned with what is known so far. GLILD patients have worse lung function in comparison to the general population and CVID non-GLILD patients, regarding both volumes and gas transfer[37]. CT scans confirmed that the main findings are ground-glass opacities, fibrosis, presence of non-perilymphatic nodules, and lymphadenopathies[23,74,75]. Biopsies (both of the lung and other organs affected by the disease) showed a prevalence of lymphocytic infiltration, granulomatous inflammation, and fibrosis[40,81].

These results seem to agree with the most recent literature on possible predictors for GLILD. In particular, we compared the parameters we identified with those proposed by several studies and confirmed that by combining two clinical manifestations (splenomegaly and autoimmune cytopenia), one lung function index (DLCO%), and one immunologic marker (CD21low%)[66], 59 out of 64 GLILD patients could have been accurately diagnosed prior to histologic confirmation, representing 92% of the total cohort.

Finally, the analysis conducted on therapeutic strategies shows that rituximab is the most used treatment for GLILD patients, mainly in monotherapy, with more than half of the treated patients (52%) showing clear signs of response; the efficacy of Rituximab monotherapy has already been reported, but literature still does not support its use as first-line treatment and our sample is the biggest cohort described, ever, of GLILD patients treated with Rituximab[94–96].

Moving to the preliminary research for potential prognostic markers, we performed a case-control analysis to assess if the severity of the clinical

phenotype could be suspected before the decline of lung function parameters and the radiological progression of interstitial lung disease occurred.

We found that patients with more severe phenotypes were older and had longer CVID diagnostic delay, suggesting that probably GLILD had more time to worsen before its diagnosis and therapy initiation. Older age also means a more remote symptom onset. Hopefully, the ongoing and progressive reduction of the CVID diagnostic delay due to more widespread disease awareness will limit its prognostic impact.

The case group also had lower IgG serum levels at CVID diagnosis and needed a higher dosage of IgRT to achieve similar, despite tendentially lower, IgTL. This further underlines the need for IgRT personalization in CVID patients, according to disease-related complications. Of note, an impact of IgRT dosage on lung function decline has been reported in a small cohort[111,112].

Focusing on serum and BALF analyses, our data, despite preliminary, seem to suggest a relationship between the degree of immune imbalance and the severity of GLILD.

In particular, if serum B and T cells subpopulation do not seem significantly different between cases and controls, BALF analysis showed that cases presented tendentially higher $CD21^{low}$ B cells % with a significantly lower CD4/CD8 T cells ratio, within the normal range. On the other hand, the control group had a significantly increased CD4/CD8 ratio, higher than the threshold considered supportive for the diagnosis of sarcoidosis[76]. Our data support what has already been observed in a small cohort, that a higher ratio could lead to a favorable disease course, closer to that occurring in most sarcoidosis patients[71].

CT scans confirmed what was already studied in the past years: patients with severe GLILD have significantly greater extension and severity of lung involvement at chest HRCT scan as compared to patients with stable disease [77]. Ground glass opacities and bronchiectasis appeared to be the most relevant findings in terms of prognostic impact. However, a centralized revision of lung

imaging by expert chest Radiologists is ongoing and will hopefully lead to further prognostic considerations.

Even ^{18}FDG-PET-CT scans gave important information: almost underlying a more extensive involvement in the case group, meaning that a more severe condition may be linked to a more spread diffusion of the disease and a greater inflammatory and dysregulated state associated with the disease.

As expected, severe patients tended to have a more critical lung function impairment in comparison to controls. Lung parameters were lower than the general population in the whole sample, but statistical significance has been achieved in FEV1 and FVC levels when comparing mild and severe GLILD patients.

Interestingly, the comparison of DLCO at the first and last available spirometry in GLILD patients showed a difference: while at the first PFT, the parameter reached high significance, at the last test it did not anymore. This might be because also controls' lung function declined over time, altering the difference between the two groups. Another reason may be found in the effectiveness of the immunosuppressive therapy used for the cases. In any case, even at the last measurement, the parameter is still very close to the significance and the DLCO value in severe patients, despite treatment, is around the 5th percentile of what is expected in the general population[102].

Finally, trying to put together all those findings potentially related to a worse prognosis, we observed that:

- As DLCO% declines, the probability of a poorer outcome rises;
- The lower the IgG levels at diagnosis, the higher the risk of a more severe disease;
- A longer diagnostic delay of CVID puts the patient more at risk of having a negative prognosis.

The combination of these variables seems to provide a moderately accurate prognostic test, with an area under the curve (AUC) of 0.901. In our opinion, after validation in larger cohorts, a score based on these components may help

clinicians to discriminate between who needs deeper examinations or not, addressing the decision on prescribing further diagnostic exams or anticipating the therapeutic intervention.

6.1 Limitations of the study

The present study has different limitations, mainly linked to the relatively small size of the sample, the retrospective nature of the study, and the difficulty of organizing a multicenter analysis. We gathered a lot of data, but some information was not complete and the follow-up CRF was not always updated. A few patients moved from or were referred to other hospitals, and the investigators were not always in possession of the complete medical records. Moreover, the design of the case and control group was made upon criteria we considered to be the best to define the mild and severe phenotype, but there may be some confounding factors (*e.g.* the eventual use of treatments for overlapping conditions; the fact that some data were gained indirectly from subsequent records, possibly being incomplete). Anyway, GLILD is a complication occurring in around 20% of CVID patients and CVID itself is a rare disease; thus, larger and more accurate studies are not easy to be done, and the ongoing prospective studies will take years to give useful results. Finally, the heterogeneity of the disease would also make it difficult to design randomized controlled interventional trials.

7. Conclusions

In this study, we presented one of the largest cohorts of GLILD patients, providing preliminary data from a national study aimed at improving the understanding of GLILD's behavior, prognostic factors, and therapeutic features. We reaffirmed that GLILD should be recognized as a multisystemic disease with a primary pulmonary focus. While lung biopsy remains a key diagnostic tool, alternative approaches, including less invasive and more peripheral histologic or clinical-radiologic methods, should also be considered. Parameters such as DLCO%, CD21low B cells%, splenomegaly, autoimmune cytopenia, and BALF cell analysis (along with microbiological differentiation in BALF) are valuable tools that, when combined with consistent HRCT findings, can help reach a diagnostic consensus. This approach may not only lead to a safer diagnostic process for patients but also allow clinicians to initiate the diagnostic workup without immediately resorting to invasive biopsy.

Additionally, we identified further evidence for the involvement of TACI and CTLA-4 in GLILD pathogenesis, potentially opening the door to novel therapeutic strategies in the future. Regarding treatment, we emphasized the role of replacement therapy and Rituximab in patient management.

Through a case-control analysis, we also examined the impact of milder versus more severe manifestations of GLILD on different pathogenic aspects. We found that patients with progressive disease experienced significant declines in lung function, along with more pronounced pathology on CT and 18FDG-PET-CT scans. These findings suggest that early detection of rapid functional decline through close monitoring of these variables could prompt earlier initiation of treatment in patients with poor prognostic indicators, considering the high mortality rate associated with GLILD.

Furthermore, we proposed the development of a prognostic score by combining various clinical, laboratory, and functional parameters (e.g., serum IgG levels, diagnostic delay of CVID, and DLCO%), which could be validated in prospective cohorts to assist clinicians.

In conclusion, while this study has addressed many aspects of GLILD, further research is required, ideally with larger cohorts and a prospective design. The ultimate goal is to establish a more standardized understanding of the disease, with a globally accepted diagnostic approach and unified management strategies, while still allowing for personalized treatment when necessary.

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