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A clinical, histological and transcriptomic characterization of a selected series of Castleman disease's cases

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PhD dissertation of
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TABLE OF CONTENTS

1. Introduction

1.1 Castleman Disease

Castleman disease (CD) identifies a group of heterogeneous lymphoproliferative disorders included by the recent WHO classification among the tumor-like lesions with B-cell predominance [1]; it consists of several subtypes with different etiologies, clinical manifestations, and histological features.

First described by Castleman et al in 1954, he reported the case of a 42-year-old man presenting with fever, weakness, cough, sweating and with a large mediastinal mass on chest X-ray. Histological examination trough bioptic specimen showed the presence of lymphoid follicles' hyperplasia and marked capillary proliferation with thick hyalinized walls [2]. Castleman then analyzed a series of other 12 patients with the same features describing their clinical and pathological characteristic [3]. The disease with the features reported by Castleman is currently the hyalinevascular-unicentric form of CD (HV-UCD) [4].

Later, in 1969 Flendrig reported the existence of a different type of CD associated with systemic symptoms and, in which, at histological examination, the plasma cells infiltrate the lymph node's interfollicular area. Thus, in 1972 two histological types were established, HV and plasma cell type (PC) [4].

In 1980 Mori et al described 10 cases with systemic lymphadenopathy, polyclonal hypergammaglobulinemia and non neoplastic plasma cell proliferation on lymph node biopsy termed as idiopathic plasmacytic lymphadenopathy (IPL) [5].

Three years later Frizzera et al defined multicentric CD (MCD) by reporting 15 cases with PC-type CD-like histology and systemic symptoms [6].

In the early 1980s, with the increase in the acquired immunodeficiency syndrome (AIDS) epidemic, it was recognized the link between Kaposi's sarcoma and MCD, which led Soulier et al in 1995 to detect Kaposi's sarcoma herpes virus/herpes virus 8 (KSHV/HHV8) sequences in all human immunodeficiency virus (HIV)-positive MCD cases and in less than half of the HIV-negative ones. Thus "KSHV/HHV8-associated MCD" was established [6].

However, not all MCD cases were correlated to HHV8 positivity. In 2017 Fajgenbaum et al developed the first international consensus diagnostic criteria for idiopathic MCD (iMCD) [7]. iMCD is an heterogeneous disease concept that can be further divided into at least 2 subtypes based on clinical presentation. One is iMCD with TAFRO (thrombocytopenia, anasarca, reticulin fibrosis, renal dysfunction, and organomegaly) (iMCD-TAFRO). iMCD without TAFRO symptoms is called iMCD-not otherwise specified (iMCD-NOS), which currently also include IPL [4,8].

MCD can occasionally co-occur with POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal plasma cell disorder and skin changes) and these cases are referred to as POEMS-associated MCD [9].

So, based on the above background, CD can clinically present with unicentric (single lymph node/lymphoid station) or multicentric lymphadenopathies. UCD patients usually have no or mild symptoms, whereas MCD patients may present symptoms of severe inflammation, frequent cytopenia, and organ dysfunction [9–11].

Based on the etiological driver, the Castleman Disease Collaborative Network (CDCN) decided to distinguish MCD into HHV8-associated MCD with or without HIV coinfection (HIV+/-); POEMS-associated MCD; and idiopathic MCD (iMCD) which is furtherly distinguished into iMCD-NOS, often associated with thrombocytosis and hypergammaglobulinemia, and iMCD-TAFRO (Figure 1) [7].

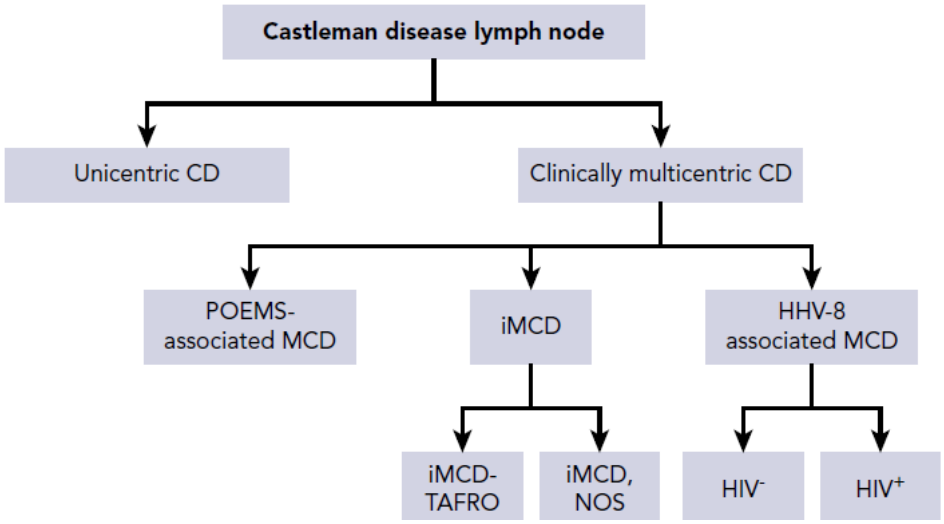

Figure 1. CD classification [7]

CD mostly occur in lymph node, extranodal localization are rare and they are mostly described in mediastinum or retroperitoneum. Subcutaneous and muscoskeletal localization are exceptional [12]; it has also been reported a pancreatic localization of the disease with 19 cases described in literature [13].

It has never been reported transition from UCD to MCD [14].

1.2 Epidemiology

All forms of CD are rare. A specific ICD-10 diagnosis code was introduced only in 2016, and diagnostic criteria for iMCD were established in 2017. Prior evaluations were based on case series and case reports. Furthermore the epidemiology of UCD differs notably from MCD. CD may present in all age-group, however UCD patients tend to be younger than MCD ones and, if affected, children are most likely to develop UCD [15,16].

1.2.1 Unicentric Castleman disease

The UCD incidence in the USA has been estimated as 16 million person-years; it can occur in individuals of all ages, with a median age of onset in the fourth decade and without gender preference [17,18].

1.2.2 Multicentric Castleman disease

MCD incidence in the USA has been estimated as 5 or 21-25 per million person-years, depending on the study, with a median age of onset in the fifth to seventh decades [17,19]. There seems to be a male preponderance [17]. All HIV positive patients have an HHV8-associated MCD whereas HHV8-associated MCD in HIV negative patients accounts for 2-50% of cases, with variations depending on the endemicity of HHV8 in different countries. HHV8 infection is indeed low in North Europe, South-East Asia and Carribeans; its prevalence reach the 10% in the Mediterrean populations and become highly prevalent in sub-Saharan Africa where it can reach 40% [9,15].

There are contrasting data about POEMS-associated MCD with prevalence from 7% [20] to 32% [21].

1.3 Risk Factors

No known risk factors exist for UCD or iMCD and there seems to be no clear associations with particular ethnicities either; in a Japanese studies no family history nor genetic predisposition were noted [15,22].

1.4 Pathogenesis

1.4.1 Unicentric Castleman disease

UCD is a benign, localized hyperplasia of lymphoid tissue originally termed as "giant lymph node hyperplasia". Its etiopathogenesis is still not well known, altough it seems to be a clonal process involving follicular dendritic cells (FDCs) as suggested by some papers, whereas no immunoglobulin heavy-chains (IGH) nor T-cell receptor (TCR) γ or β rearrangements have been found [14,23,24]. Supporting this hypothesis UCD can present FDCs prominence/displasia and, in very rare cases, can progress to FDCs sarcoma [14].

FDCs are essential for the growth of the germinal center (GC) since they direct lymphocytes in the correct lymph node regions through chemokines secretion, like CXCL13, and they promote B-cells survival [25,26]. Other cytokines and adhesion molecules that may have a role are vascular endothelial growth factor (VEGF), ICAM1 e CD54 [27]. Furthermore, studies of the transcriptome in CD tissue revealed expression and upregulation of markers for FDCs, angiogenetic factors, extracellular matrix remodelling factors, complement components, and markers for germinal center activation [28,29].

Mutations in *PDGFRB* have been found in 17% of UCD patients and this mutation has been observed in CD45 negative stromal cells by in situ hybridization (ISH), these mutations are thought to confer proliferation and survival advantages to stromal cells, thus reinforcing the hypothesis of stromal-cell derived neoplasia [30]. No associations with viral infection have been found [31].

Abnormalities within mytogen-activated protein kinase (MAPK) and interleukin signaling pathways have also been recognized [32].

The above alterations have been found mostly in HV-UCD whereas PC-UCD might be associated to interleukine 6 (IL-6) overproduction, because of its similarities to MCD. Furthermore PC-UCD has some morphological similarities with autoimmune disease's lymph node alterations, so it may start as an autoimmune reaction pattern [27].

1.4.2 Multicentric Castleman disease

HHV8-associated MCD

HHV8 is a γ-herpesvirus that may infect various cells including endothelial cells, B cells, and antigen-presenting cells. It is the cause of HHV8-associated MCD which is a polyclonal proliferation that occur in individual HHV8-infected and HIV positive or negative. Like Epstein-Barr virus (EBV), HHV8 exhibit the latent and the lytic phases characteristic of all herpesviruses. During the latent phase the virus is dormant and expresses very few latent viral genes; instead, during the lytic phase, the virus expresses multiple lytic proteins that help in viral replication and in the production of virions. Both latent and lytic HHV8-viral

proteins can be found in HHV8-associated MCD lymph nodes, like the HHV8 latency-associated nuclear antigen (LANA) and the lytic viral proteins encoded by viral IL-6 (vIL-6).

vIL-6 is a homologue of cellular human IL-6 (hIL-6) that only needs to bind one of the two IL-6 receptor subunits to exerts its effect [33,34], thus it has a broader spectrum of target cells and probably induces the "cytokine storms" characteristic of HHV8-associated MCD. The infected cells are mainly plasma cells or plasmablasts located in the mantle zone of lymphoid follicles; some studies reported vIL-6 expression in HHV8-associated MCD to be highly variable both in the same individual and between individuals [15,16].

However, the clinic is not explainded by vIL-6 alone; HHV8-associated MCD is also characterized by excessive expression of human cytokines like hIL-6, IL-10 and other inflammatory cytokines; their increased expression may be driven by the virus. For example, LANA can upregulate hIL-6 through a variety of different mechanisms. Furthermore, in HHV8-associated MCD, hIL-6 and vIL-6 function in a paracrine fashion because vIL-6 upregulate VEGF, similar to hIL-6; VEGF is a potent angiogenetic factor that further induces hIL-6 expression from endothelial cells, thereby promoting proliferation of plasmablasts [15,35,36].

Another important factor in HHV8-associated MCD pathogenesis are T-cells; unlike Kaposi's sarcoma (KS), HHV8-associated MCD is not associated with a deficiency of HHV8-specific CD8+ T cells or a limitation in their functional profile [37]. Newer data indicate also a crucial role of invariant natural killer T cells (iNKT cells) which are essential for the control of infected B-cells. Their alterations in HHV8-associated MCD appeared to be independent of HIV infection [16,37,38].

POEMS-associated MCD

The pathogenesis of POEMS-associated MCD has been linked to cytokine production by monoclonal plasma cells. The cytokine which better correlate with the disease acticity is VEGF, other pathogenetic cytokines appeared to be IL-6, IL-1, IL-2, IL-1beta and tumor necrosis factor-alfa (TNF α) [14,27].

iMCD

In iMCD patients the disease etiology is still unknown. Candidate etiologic driver are: uncontrolled infection by an unidentified virus (pathogen hypothesis); autoantibodies, or autoreactive T cells in conjunction with germline mutations (autoimmune hypothesis); germline mutations in genes regulating inflammation (autoinflammatory hypothesis); and/or somatic mutations in monoclonal lymph node cells leading to ectopic cytokine secretion (paraneoplastic hypothesis) [14,39]. Next-generation sequencing (NGS) has identified several cases of iMCD with somatic alterations or germline variants, but the alterations are not consistent across patients and their role is unknown [40–43].

Whatever may be the cause, it is clear that a cytokine storm involving IL-6 is crucial to iMCD pathogenesis [27] (Figure 2). Other cytokines implicated in iMCD pathogenesis are VEGF, IL-1, IL-2, CXCL13 and TNF

[44–46]. Regarding CXCL13 recent transcriptomic and proteomic studies validated CXCL13 as the protein most prominently up-regulated in iMCD [28,29,47].

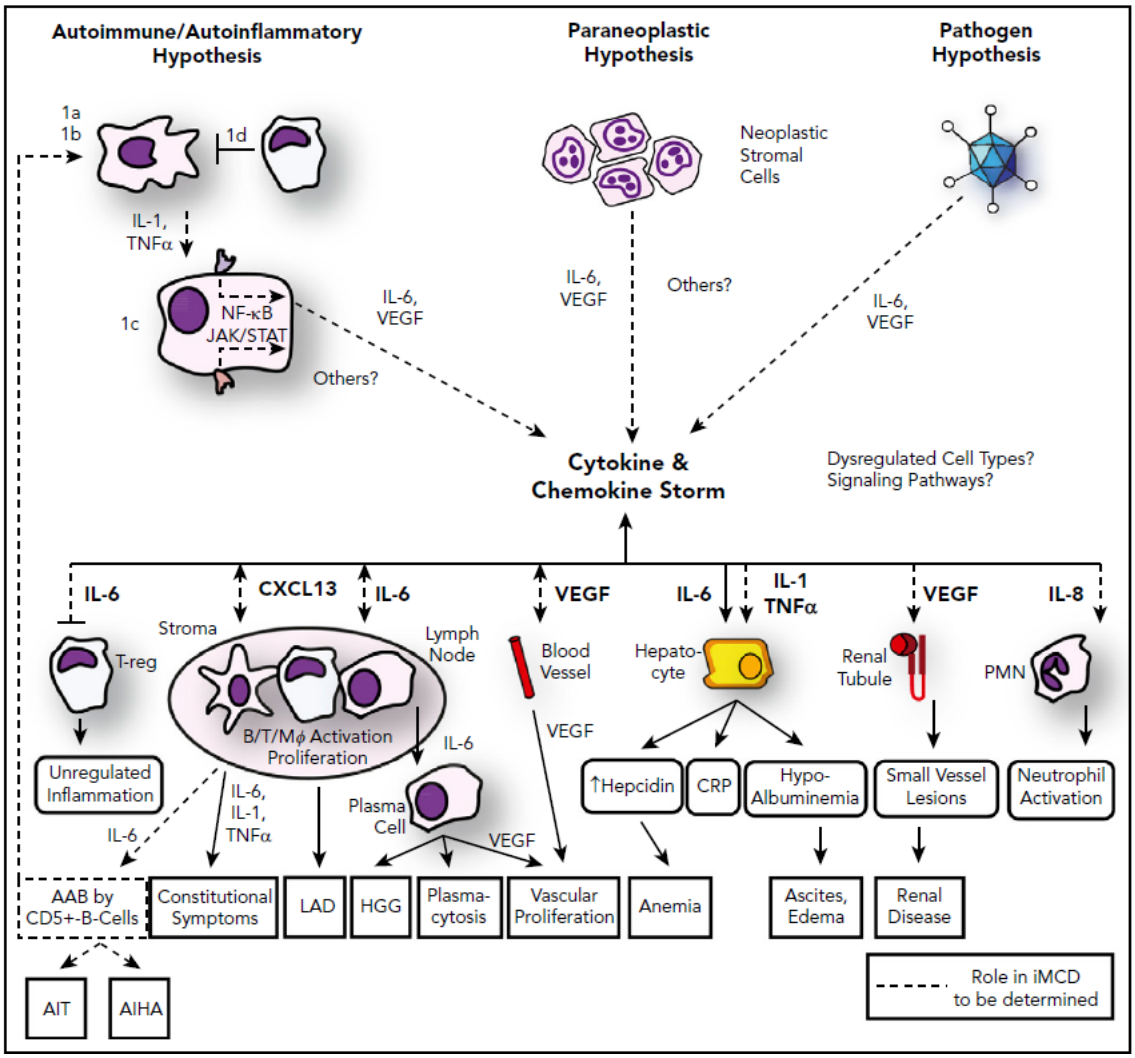

Figure 2. Possible pathogenetic models for iMCD [48]

Other mechanism that may be involved include T cell activation and JAK/STAT, PI3K/Akt/mTOR and type I interferon signalling pathways. JAK/STAT is an important signaling pathway through which IL-6 exerts its effects intracellularly; several studies have recently indicated a dysregulation of the JAK/STAT pathway in iMCD. Activation of mTOR pathway stimulates the biosynthesis of proteins which influences cell growth, proliferation, angiogenesis, and apoptosis. Its genetic alterations and increased activity have been demonstrated in various tumor entities. Moreover, a type I interferon response was identified as a common gene signature upregulated during iMCD flares [49–55]. In iMCD, genes affecting chromatin organization are more commonly observed [56]. About IGH or TCR rearrangements, IGH rearrangements have been found only in a small subset of MCD patients, and in the majority of these cases the patients also had a lymphoma. TCR rearrangements are even more rare [24,57,58].

Finally, in iMCD the presence or absence of mutations in genes associated with auto-inflammatory disease has been recently reported to possible modulate clinical symptoms and inflammasome activity may be linked to disease progression [59–61].

1.5 IL-6 role in Castleman disease

IL-6 is a 184-amino acid protein cytokine that is produced by many types of cells (T cells, B cells, macrophages, endothelial cells, dendritic cells, epithelial cells, etc…) and is expressed during states of cellular stress, such as inflammation, infection, wound sites, and cancer. IL-6 levels may increase several thousand-fold in these states and may help to coordinate the response to dysregulation of tissue homeostasis. IL-6 acts through a membrane bound IL-6 receptor (IL-6R) which together with another receptor (glycoprotein 130, gp130) leads to intracellular signalling initiation. IL-6 R is expressed on only a few types of cells, however it is also produced as a soluble, secreted protein that with IL-6 can stimulate all gp130-expressing cells by IL-6 trans-signaling. IL-6 is important for both the adaptive and innate immune system and for mesenchymal and stromal responses during inflammation. It promotes the develpment of pathogenic T-helper 17 cells and the maturation of B lymphocytes. Innate immune cells like neutrophils, and monocytes/macrophages produce and respond to IL-6, resulting in autocrine feedback loops that amplify inflammation.

IL-6 production is now a known key driver in CD development and most of CD systemic symptoms are linked to IL-6 hyperfunction (Figure 3) [62].

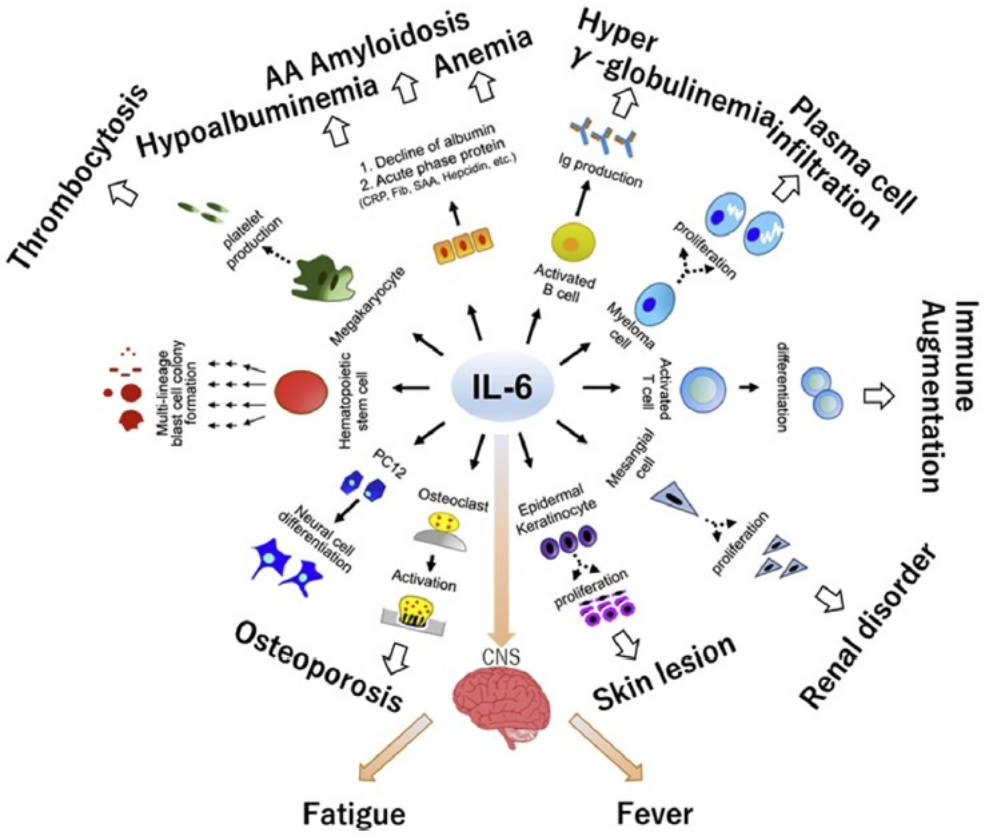

Figure 3. IL-6 functions and symptoms linked to its overfunction [62].

1.6 Clinical features

CD clinical spectrum is broad, with UCD usually presenting as a solitary, asymptomatic lymph node enlargement; in contrast, iMCD ranges from mild constitutional symptoms to multiorgan failure. HHV8-associated MCD often presents as a severe disease with relapsing course that may be fatal if left untreated [16].

1.6.1 Unicentric Castleman disease

UCD is often an incidental finding, with symptomps, when present, due to the localization or compression of nerves or vessels. Asymptomatic UCD lesions are sometimes identified by imaging tests performed for other purposes. Systemic constitutional symptoms (i.e. weight loss, fatigue) are uncommon and usually not severe if present; similarly laboratory abnormalities are rare [18,63].

To make a diagnosis of UCD, lymph node swelling must be confined to a single lymph node or node station. The involved lymph node is typically larger than enlarged nodes in MCD, with a median size of 5,5 cm. All lymph node regions may be affected but in most cases it tends to be a solitary mass in the mediastinum. Other common presentation sites are neck, abdomen, retroperitoneum and mesentery [64,65].

The diagnostic work up must include radiological examinations; X-ray may be used if the disease is localized in the mediastinum but, usually, it is not useful in abdominal or pelvic cases [18].

Most commonly whole-body computed tomography (CT) is used or computed tomography-fluorodesossiglucose-positron emission tomography (CT-FDG-PET) [66]. Magnetic resonance imaging (MRI) can be useful to show the disease extension and to clarify the connection with adiacent structure. CT-FDG-PET give information about the metabolic state; the standardized uptake value (SUV) is lower in CD than lymphomas [9,18,66].

However, to reach the diagnosis of CD is important to perform histological examination of an excised lymph node [18].

1.6.2 Multicentric Castleman disease

All forms of MCD are characterized by a clinical presentation of systemic inflammatory symptoms, associated organ system dysfunction and laboratory abnormalities.

MCD patients present with generalized lymphadenopathy, splenomegaly and sometimes hepatomegaly. Systemic symptoms include fever, weight loss, anasarca; anemia is common as is hypoalbuminaemia, almost all cases present elevations in inflammatory markers. The inflammatory symptoms may be intermittent, occurring in flares; sometimes, in severe case, when the inflammatory flare is established, it becomes self sustaining and may be life-threatening [7,9,10,27,67].

In MCD mediastinal involvement is less frequent than UCD [10,68].

For the diagnosis is also important to evaluate HIV and HHV8 virological status to distinguish between iMCD and HHV8-associated MCD; HHV8 status must be analyzed both in lymph node by immunohistochemisty (IHC) and in serum by polymerase chain reaction (PCR) analysis [9,69].

It is also important to do a neurologic evaluation, serum and urine elettrophoresis to look into the possibility of POEMS syndrome [9].

HHV8-associated MCD

HHV8-associated MCD is most commonly seen in HIV positive patients, its incidence increased over time due to the introduction of antiretroviral therapies [70].

Typically, the disease progresses in flares that last several days, they are followed by oligosymptomatic and asymptomatic periods of several weeks. During flares, most common symptoms are fever, weekness, night sweats, and weight loss. There are also high levels of C reactive protein, severe anemia, hypoalbuminemia, thrombocytopenia. Sometimes an hemophagocytic lymphohistiocytosis can occur. Usually, symptoms fluctuates without any intervention but self-limiting courses are rare; with increasing duration of the disease, the frequency of flares can increase [15,27].

There are no known correlation between HIV viral load or CD4+ T cells count and disease activity [71].

Kaposi's sarcoma can coexhist with MCD in both HIV positive and negative patients, and there is a significantly increased risk of malignant lymphomas, including rare subtypes such as plasmoblastic lymphoma and primary effusion lymphoma [27].

POEMS-associated MCD

Sometimes, although rarely, MCD is associated with POEMS syndrome. POEMS syndrome is a rare paraneoplastic disease associated with a plasma cell neoplasia. Diagnostic criteria for POEMS syndrome are reported in Table 1. Diagnosis is confirmed when both mandatory criteria, 1 of the 3 major criteria and 1 of the 6 minor criteria are present [72,73].

Table 1. POEMS syndrome diagnostic criteria [72,73].

Mandatory major criteria	1. Polyneuropathy (typically demyelinating) 2. Monoclonal plasma cell-proliferative disorder (almost always λ)
Other major criteria (one required)	3. Castleman disease 4. Sclerotic bone lesions 5. Vascular endothelial growth factor elevation
Minor criteria	6. Organomegaly (splenomegaly, hepatomegaly, or lymphadenopathy) 7. Extravascular volume overload (edema, pleural effusion, or ascites) 8. Endocrinopathy (adrenal, thyroid, pituitary, gonadal, parathyroid, pancreatic) 9. Skin changes (hyperpigmentation, hypertrichosis, glomeruloid hemangiomata, plethora, acrocyanosis, flushing, white nails) 10. Papilledema 11. Thrombocytosis/polycythemia
Other symptoms and signs	Clubbing, weight loss, hyperhidrosis, pulmonary hypertension/restrictive lung disease, thrombotic diatheses, diarrhea, low vitamin B_{12} values

An important clinical feature is peripheral polyneuropathy, probably caused by VEGF, which should raise suspicion of a POEMS-associated MCD. This also applies to cutaneous hyperpigmentations, which must be distinguished from cherry hemangiomas [16].

iMCD

iMCD can be quite variable in terms of severity, course, and symptoms. The most common symptomps are lymph node swelling, anemia, and fever. Symptomatology are often less pronounced in iMCD compared to HHV8-associated MCD [9,15,27]. Hemangiomatous skin lesions can occur ("cherry hemangiomatosis") during active phases of the disease with fluctuaction in size; polyneuropathies are uncommon [16].

iMCD is also associated with significant comorbidities that may limit the overall prognosis.

A multidisciplinary approach is important for the diagnosis of iMCD because other disease may mimic it, thus, for the diagnosis are required different inclusion and exclusion criteria (Table 2 and 3).

Table 2. iMCD diagnostic criteria [7,9].

Inclusion criteria	Exclusion criteria
I. Major criteria (need both)	**Infection-related disorders**
1. Histopathologic lymph node	1. HHV8
2. Enlarged lymph nodes in ≥2 lymph node stations	2. EBV LPD
II. Minor criteria (need ≥2 of 11 with ≥1 laboratory criterion)	3. Inflammation and adenopathy by other infection
Laboratory	**Autoimmune/inflammatory disease**
1. Elevated ESR or CRP	1. SLE
2. Anemia	2. Rheumatoid arthritis
3. Thrombocytopenia/tosis	3. Adult-onset Still disease
4. Renal dysfunction or proteinuria	4. Juvenile idiopathic arthritis
5. Polyclonal hypergammaglobulinemia	5. Autoimmune LPS
6. Hypoalbuminemia	**Malignant LPD**
Clinical	1. Lymphoma
1. Constitutional symptoms	2. Multiple myeloma
2. Large spleen and/or liver	3. Primary lymph node plasmacytoma
3. Fluid accumulation	4. FDC sarcoma
4. Eruptive cherry angiomata or violaceous papules	5. POEMS syndrome
5. Lymphocytic interstitial pneumonitis	

Table 3. Other features supporting iMCD diagnosis [7].

Elevated IL-6, sIL-2R, VEGF, IgA, IgE, LDH, and/or B2M
Reticulin fibrosis of bone marrow (particularly in patients with TAFRO syndrome)
Diagnosis of disorders that have been associated with iMCD: paraneoplastic pemphigus, bronchiolitis obliterans organizing pneumonia, autoimmune cytopenias, polyneuropathy (without diagnosing POEMS‡), glomerular nephropathy, inflammatory myofibroblastic tumor

iMCD is also subdivided based on clinical presentation in iMCD-TAFRO and iMCD-NOS.

iMCD-NOS

These patients tend to have elevated platelet counts, very elevated immunoglobulin levels and PC histopathological features. They usually also have a less aggressive clinical course with a better response to corticosteroid therapy [9,74,75].

iMCD-TAFRO

Firstly described in Japan; the average age of patients with TAFRO is 50-59 years, without gender preference. TAFRO syndrome has an acute to subacute clinical course and sometimes can be fatal. iMCD-TAFRO patients have a more aggressive clinical course than iMCD-NOS with thrombocytopenia, ascites, fever, reticulin fibrosis and organomegaly; they also tend to have normal immunoglobulin levels and mixed or HV histopathological features [15,76,77].

Diagnostic criteria for iMCD-TAFRO are reported in Table 4.

Table 4. Diagnostic criteria for iMCD-TAFRO [9,76].

Criteria
Histopathological criteria: need all
Typical LN pathology (atrophic GCs with enlarged nuclei of ECs, proliferation of endothelial venules, small numbers of mature PCs)
Negative LANA-1 for HHV8
Major criteria: need 3 of 5
Thrombocytopenia (<100 000/μL)
Anasarca (pleural effusions and ascites on CT)
Fever (>38°C)
Reticulin fibrosis
Organomegaly
Minor criteria: at least 1
Hyper/normoplasia of megakaryocytes
High alkaline phosphatase without markedly elevated transaminases

1.7 Histological features

Histopathologic descriptions of CD include a hyaline-vascular subtype (HV) when clinically presenting as unicentric disease, a hypervascular subtype (HyperV) when presenting as multicentric disease, a plasma cell (or plasmacytic) subtype, and mixed forms that are in between [63].

1.7.1 Unicentric Castleman disease

The HV variant is the most common histological type of UCD, representing 65-75% of all cases [32].

The histological features can be divided into two groups: follicles modifications, and stroma-modification [32].

HV is characterized by capsular fibrosis around lymphoid follicles with broad fibrous bands disrupting the normal architecture. Abnormal follicles are present with small or atrophic germinal centers (GC) and sclerotic arterioles radially penetrating into GC ("lollipop lesions"); mantle zones are hyperplastic and composed of concentric rings of small lymphocytes ("onion skin changes"); sometimes the same mantle zone contain 2 or more small GC ("twinning", Figure 4) [32,78–80]. GC are characteristically depleted of B cells, with hyaline deposits and FDCs that sometimes can be dysplastic [81].

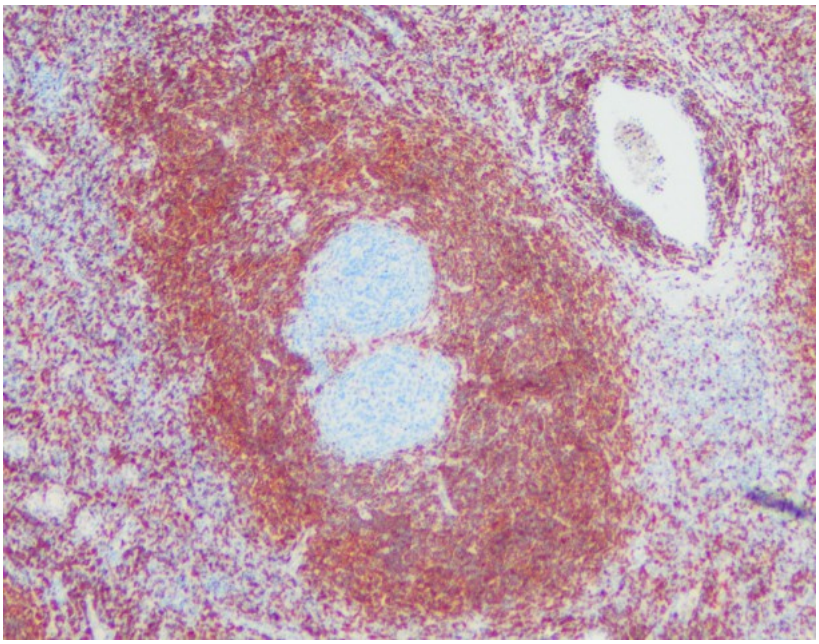

Figure 4. "Twinning"

The interfollicular areas are composed by numerous high-endothelial vessels, interfollicular proliferations of stromal cells including FDCs and small foci of hyperplastic plasmacytoid dendritic cells [79,80].

Variations in the proportions of the follicular and interfollicular components can result in a wide spectrum of appearances, ranging from predominantly follicular (follicular modifications >50% of the alterations) to stroma-rich (interfollicular features>50%) presentations [27,80].

Obliterations of the subcapsular and medullary sinuses is also seen, and often is one of the earliest manifestations of UCD [32].

PC-UCD represents 25% of all UCD cases. It is characterized by follicular hyperplasia with reactive GC, clusters or sheets of plasma cells in the interfollicular areas, and overall preserved architecture. The plasma cells are most often polytypic and can show a wide range of cytologic features. Vascular proliferation is variable, and sinuses are present. Even though, most follicles are hyperplastic a small subset can show HV changes; as the "onion skin" mantle zone can be seen in some cases and the penetrating blood vessels in GC [27,32,80]. Hybrid cases with features of both variants (HV and PC) can be observed, generating the so called mixed form [63].

1.7.2 Multicentric Castleman disease

HHV8-associated MCD

The pathological features of HHV8-associated MCD are usually those of the PC or mixed-type with some differences. The key distinction is the presence of HHV8-infected cells; they are plasmacytic-plasmablastic B cells predominantly seen in the mantle zones and centers of the follicles, but they can also be seen as single cells in the interfollicular area. In addition there is a proliferation of polyclonal but monotypic IgMλ restricted plasmocytoid cells, a proportion of which are HHV8+ cells as demonstrated by the expression of LANA1. The infected cells can proliferate as large cells (plasmablasts) and form small clusters (microlymphoma) or confluent sheets (frank lymphoma). Of note, in the HHV8-associated MCD affected lymph node can co-occur a Kaposi's sarcoma [82–87]. Bone marrow changes in HHV8-associated MCD in HIV positive patients, predominantly consist of reactive plasmacytosis ascribed to increased IL-6 levels coupled with the presence of lymphoid follicles exhibiting the typical changes of Castleman disease (although this second feature is actually quite rare); in addition scattered HHV8/LANA1+ cells, and increased amount of histiocytes with sporadic hemophagocytosis can be found [88,89].

POEMS-associated MCD

POEMS-associated MCD usually presents a PC-type histological features with a few regressed GC [27].

iMCD

In 2017 a consensus work of pathologists and clinicians expert in CD established diagnostic criteria for iMCD (Figures 5 and 6). The panel defined the spectrum of iMCD histopatological features as follows: cases with regressed GC and prominent vascularization were considered to fall on the hypervascular end of the spectrum, hyperplastic GC with prominent plasmacytosis were considered to fall on the PC end of the spectrum, and patients with overlapping features of both represented mixed histopathology. The pathological findings were also incorporated into consensus diagnostic criteria for iMCD (Table 2) [7,9].

This panel decided to alter the nomenclature within the iMCD group from HV to hypervascular (HyperV) because pathologists often associate the term “hyaline vascular” with UCD. Although the HyperV subtype exhibits histological features of the HV subtype, such as marked vascular proliferation and atrophic GC, in the HyperV subtype, the nodal architecture is less distorted and FDCs dysplasia is less frequently observed [7,9,27].

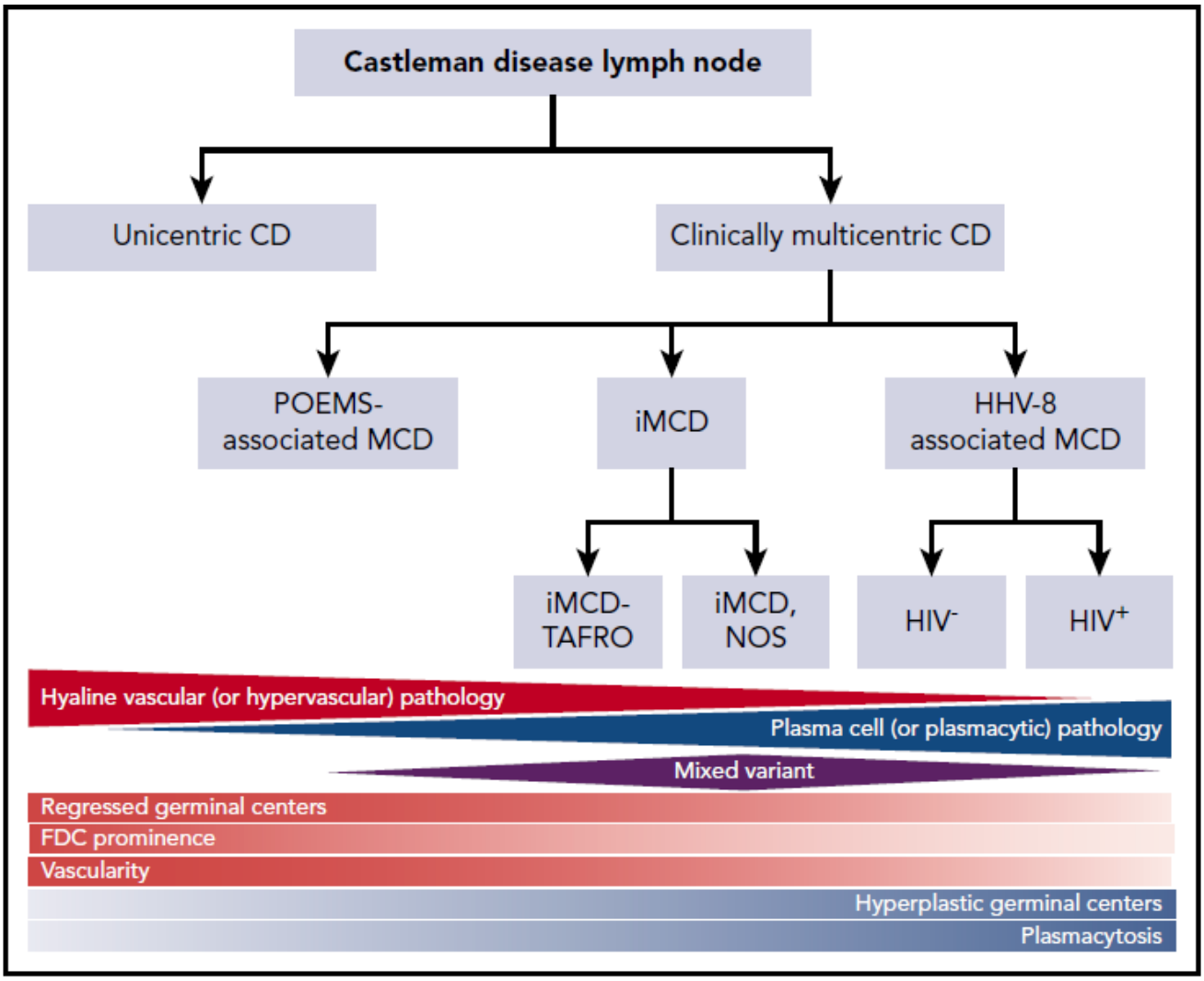

Figure 5. CD Classification based on clinical, etiological and histological features [9].

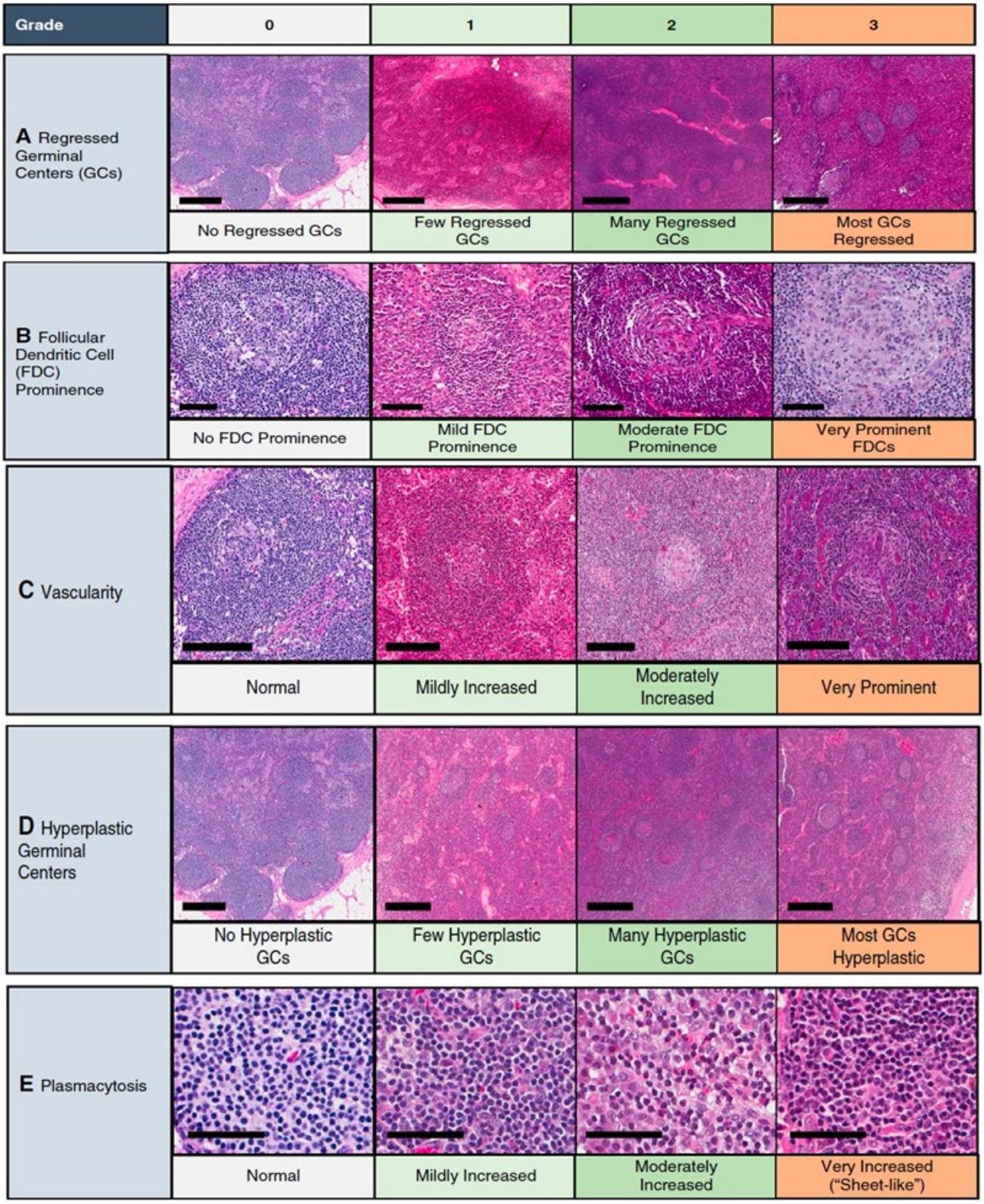

Figure 6. Grading of iMCD's histological features [7].

About bone marrow features in iMCD a recent article by Belyaeva et al revealed a high proportion of cases with hypercellularity, megakaryocytic atipia, reticulin fibrosis, and plasmacytosis in patients with iMCD-NOS and iMCD-TAFRO, with significantly more megakaryocytic hyperplasia in iMCD-TAFRO; these findings, however, are relatively nonspecific and they can be seen in various other diseases [90–92].

1.8 Transcriptome and cytokine microenvironment of CD

In recent years two studies about transcriptome and cytokine microenvironment in UCD and MCD lymph node have been published. These investigations have documented, in UCD cases, the expression and up-regulation of FDCs markers like CXCL13, angiogenetic factors, extracellular matrix remodeling factors, complement components, and markers for GC activation. MCD instead showed up-regulation of IL-2, plasma cell differentiation, FDCs maker (CXCL13), fibroblastic reticular cell cytokine, angiogenetic factor, and mTORC1 pathway genes [28,29].

IL-6 findings were contrasting in the 2 study, because whereas Wing et al found an up-regulation of IL-6 in MCD lymph node this was not confirmed by Horna et al [28,29]. However, these findings point out that the nodal microenvironment, including its immune cellular components, plays a crucial pathogenetic role in CD.

1.9 Differential diagnosis

CD histopathological features are not enough to made a diagnosis of CD alone, because they might be found in diverse disease entity [63].

Differential diagnosis in UCD cases, HV, PC or mixed, comprehends different diseases from infectious one (toxoplasmosis, syphilis etc..), to autoimmune diseases like systemic lupus erythematosus (SLE) to lymphomas (low grade non-Hodgkin lymphomas, Hodgkin lymphomas, T-cell lymphoma) and plasma cell neoplasms or proliferations of stromal components (FDCs, vascular or smooth muscle proliferations) [63].

In MCD differential diagnosis includes autoimmune disorders like SLE, reumathoid arthritis and autoimmune lymphoproliferative syndrome, B-cell and T-cell lymphomas and plasma cell neoplasm [9,27,63].

Lastly, a very difficult differential diagnosis can be with IgG4-related disease (IgG4-RD).

IgG4-RD is a clinical entit,y first described in 2001 in Japan, that can affect different organs. The clinical symptoms of IgG4-RD are dependent on the affected organs, the disease can be severe if there are serious complications such as obstruction or compression symptoms due to organomegaly or organ dysfunction caused by cellular infiltration or fibrosis. Since several problems in clinical practice have arisen during the years after the first description, the Japan IgG4 team updated the 2011 diagnostic criteria for IgG4-RD, which consists of 3 domains (Table 5): - clinical and radiological features; -serological diagnosis; - pathological diagnosis which is composed by 3 sub-items including storiform fibrosis and obliterative phlebitis [93–95].

Table 5. The 2020 revised diagnostic criteria for IgG4-RD [94]

[Item 1] clinical and radiological features
One or more organs show diffuse or localized swelling or a mass or nodule characteristic of IgG4-RD. In single organ involvement, lymph node swelling is omitted.
[Item 2] serological diagnosis
Serum IgG4 levels greater than 135 mg/dl.
[Item 3] pathological diagnosis
Positivity for two of the following three criteria:
① Dense lymphocyte and plasma cell infiltration with fibrosis.
② Ratio of IgG4-positive plasma cells /IgG-positive cells greater than 40% and the number of IgG4-positive plasma cells greater than 10 per high powered field
③Typical tissue fibrosis, particularly storiform fibrosis, or obliterative phlebitis
Diagnosis:
Definite: 1) +2) +3)
Probable: 1) +3):
Possible: 1) +2)

Explanatory note 1: Combination of organ-specific diagnostic criteria*.
Patients with a possible or probable diagnosis by comprehensive diagnostic criteria who fulfill the organ-specific criteria for IgG4-RD are regarded as being definite for IgG4-RD.
*Diagnostic criteria according to the IgG4-related organ:.
① International consensus diagnostic criteria for autoimmune pancreatitis[7], ② IgG4-related lacrimal gland, saliva adenitis diagnostic criteria[8], ③ Diagnostic criteria for IgG4-related kidney disease[9], ④ Clinical diagnostic criteria of IgG4-related sclerosing cholangitis 2012[10], ⑤Diagnostic criteria for IgG4-related ophthalmic disease[11], ⑥Diagnostic criteria for IgG4-related respiratory disease[12], ⑦ Diagnostic criteria for IgG4-related large periarteritis/periarteritis and retroperitoneal fibrosis[13].
Explanatory note 2: exclusion diagnosis.
1) It is important to acquire tissue samples from each involved organ to distinguish malignant tumors (e.g. cancer, malignant lymphoma) and similar benign conditions (e.g. Sjögren syndrome, primary sclerosing cholangitis, multicentric Castleman's disease, secondary retroperitoneal fibrosis, granulomatosis with polyangiitis, sarcoidosis, eosinophilic granulomatosis with polyangiitis).
2) It is important to exclude an infectious- or inflammation-related disease in patients with high fever, highly elevated CRP and neutrophilia.
Explanatory note 3: pathologic diagnosis.
1) The numbers of IgG4-positive cells are usually more abundant in resected organs and partially enucleated tissue than in tissue samples obtained by needle biopsy or endoscopic biopsy. Thus, it is important to not be too particular about cell number and to provide a precise judgment.
2) Storiform fibrosis is defined as spindle-shaped cells, inflammatory cells and fine collagen fibers forming a flowing arrangement. Obliterative phlebitis is defined as fibrous venous obliteration with inflammatory cells. Both are helpful for a diagnosis of IgG4-RD. ① and ③ without ② can only be applied in a case with poor IgG4 and/or IgG staining.
Explanatory note 4: steroid reactivity.
Steroid trial is not recommended. However, if patients do not respond to initial steroid therapy, the diagnosis should be reconsidered.

However, the challenge in distinguish IgG4-RD and its mimickers, among which there is also CD, still remain, even after the introduction of the 2020 revised criteria, because some non-IgG4-RDs may meet the diagnostic criteria of IgG4-RD and thus be misdiagnosed as such. Accurate diagnosis is crucial because IgG4-RD treatments and those for other diseases are different. Because of that Satou et al proposed exclusion criteria for IgG4-RD (Table 6) [95]

Table 6. Exclusion criteria for IgG4-RD [95]

Cases that are present with any one of the clinical or pathological findings listed below cannot be categorized as definite IgG4-RD, although they may meet the diagnostic criteria for IgG4-RD

Clinical findings
- Continuing elevated serum level of CRP (≧1.0 mg/dL)†
- Elevated serum level of IgA‡
- Elevated serum level of IgM‡

Pathological findings
- Sheet-like proliferation pattern of mature plasma cells
- High degree of hemosiderin deposition
- Neutrophilic infiltration

†Continuing elevation of uncertain cause.
‡Serum level above normal range is defined as 'elevated'. Reference value of each institution should be applied.

1.10 CD and other diseases

CD may be associated with autoimmune disorders or with other diseases such as pulmonary hypertension, glomerulopathies and skin changes. Among the various diseases associated with CD there are: paraneoplastic pemphigus, hemophagocytic lynphohistiocytosis, autoimmune citopenia, peripheral polineuropathy, glomerulopaties, amyloidosis, connectivitis, LES, neoplasia [63,96,97].

Paraneoplastic pemphigus (PNP)

PNP is a mucocutaneous disorder characterized by the presence of specific autoantibodies against epidermal proteins, usually in a lymphoma context. HV/HyperV-CD is the CD subtype more commonly associated with PNP [96,98,99].

The severity of the cutaneous disease correlates with pulmonary involvement. The pulmonary manifestations include dispnea, hypoxemia, obliterans bronchiolitis, and obstructive respiratory disorders [20,63].

Hemophagogytic lymphohistiocytosis

MCD, mostly HHV8-associated MCD can present at the onset or during a second flare with hemophagogytic lymphohistiocytosis. In these cases patients also present hypofibrinogemia, high lactate dehydrogenase (LDH), high triglycerides and possible multiorgan failure [100].

Monitoring IL-10 and HHV8 viral load are important clinical parameter for monitoring disease activity [101].

Peripheral polineurophaty

Demielinating peripheral neuropathy can be observed in CD. The disease pathophisiology is still unknown; however, if present, a POEMS syndrome must be considered [102].

Amyloidosis

Amyloidosis includes a group of rare diseases characterized by deposits of amyloidogenic insoluble fibers in the extracellular space of various tissues and organs. Amyloidosis subtypes are divided based on the precursor protein that cause the disease; among them there is AA amyloidosis which is caused by deposition of serum amyloid A protein (SAA), an acute-phase protein which is normally soluble and whose plasma concentration is highest during inflammation. AA amyloidosis is a complication of a number of inflammatory diseases and infections, including, even if uncommonly, CD [103]. However, amyloidosis seems to be the most common cause of renal disease in CD [104,105].

Neoplasia

CD patients are more at risk of develop secondary neoplasia [63].

UCD patients can develop FDCs sarcomas and lymphomas (both Hodgkin and non Hodgkin lymphomas) [9,10,21].

HIV positive patients with HHV8-associated MCD are more at risk of developing lymphomas than HIV positive cases without CD [106].

HHV8-associated MCD HIV negative can develop lymphomas such as diffuse large B cell lymphoma or primary effusion lymphoma, and Kaposi's sarcoma [27,107].

iMCD patients are also at risk of developing secondary neoplasms, mostly lymphomas such as Hodgkin lymphoma, diffuse B cell lymphoma and peripheral T cell lymphoma [108].

1.11 Prognosis and Predictive factors

In UCD prognosis is usually good without a change in life expentancy; however patients may have an increased risk of developing paraneoplastic pemphigus and lymphomas [109]. According to a systematic review, after 10 years only 5% of UCD patients had died from the disease [64].

MCD cases had usually a worse prognosis than UCD, even though it has improved significantly thanks to new treatments. A study combining USA and Chinese data determined a risk score from 5 independent predictors of overall survival (OS) (age>40 years, PC subtype, hepatosplenomegaly, hemoglobin<8g/dL, and pleural effusion), its predictive value was then validated in an independent group of additional patients [110]. A recent study by Pierson et al suggested that CXCL13 can be a predictive biomarker of response to therapy with siltuximab in iMCD [47].

HHV8-associated MCD has a worse prognosis tha iMCD, however the prognosis is also expected to improve significantly with antiretroviral therapies and rituximab [16,71].

1.12 Treatments

The treatment of CD patients depends on the disease subtype; consensus has emerged that curative surgery is the gold standard for UCD and monoclonal antibody-based immunotherapy is MCD's standard of care [15].

1.12.1 Unicentric Castleman disease

Complete surgical excision is the optimal therapy for UCD when applicable as described by Castleman and confirmed 65 years later by International consensus evidence-based guidelines [3,111]. The great majority of UCD cases manageable with complete surgical excision lead to an OS of >90% at 5 years with resolution of all signs and symptoms. Relapse following complete resection is rare and paraneoplastic complications usually resolve gradually [112–114].

In selected cases, if UCD is unresectable due to size and/or location, radiotherapy can be effective [112]. Others therapeutic approaches can be embolization or neoadiuvant therapies with rituximab or siltuximab [64,111,115].

A recent series of 71 UCD patients showed that 11 patients with asymptomatic unresectable UCD remained stable with active surveillance only [115].

1.12.2 Multicentric Castleman disease

Oksenhendler et all reported MCD's OS at 5 years as follows: 100% for iMCD (only 2/27 were iMCD-TAFRO), 89% per HIVnegative HHV8-associated MCD, 65% per HIVpositive HHV8-associated MCD [10]. However the clinical management of HHV8-associated MCD and iMCD are completely different [15].

HHV8-associated MCD

The treatment of HHV8-associated MCD is based on rituximab, a monoclonal antibody against CD20 because it eliminates the viral load and reduces lymphoma's risk. Patients with life-threatening organ failure, poor performance status and co-occurrent Kaposi's sarcoma require the addition of chemotherapy. In patients with life-thretening disease, etoposide is the most frequently used chemotherapy. This rituximab-based approach showed high response rates and good long-term survival but relapse are common and usually salvageable therapy is necessary. HIV positive patients also need antiretroviral therapy. Siltuximab-based therapy is not effective in these patients because it can not bind vIL-6 [15,33,116].

POEMS-associated MCD

The treatment strategy is based on POEMS therapy and treatments can varies from localized radiotherapy to multiple myeloma approaches, including autologous haematopoietic stem cell transplantation [9].

iMCD

iMCD treatment depends on the disease's gravity (Table 7, a diagnosis of severe iMCD is done if patients have 2 out of 5 of the established criteria) [117].

Table 7. Severe iMCD grading [117].

Severe iMCD
• ECOG ≥ 2
• Stage IV renal dysfunction (eGFR < 30; Creatinine >3.0)
• Anasarca and/or ascites and/or pleural/pericardial effusion (effects of hypercytokinemia/low albumin)
• Hemoglobin ≤ 8.0g/dL
• Pulmonary involvement /interstitial pneumonitis w/dyspnea

In 2018 van Rhee et al developed guidelines for iMCD's management, however, both in severe and non severe iMCD, treatment is based on siltuximab, a monoclonal antibody against IL-6 (Figure 7) [117].

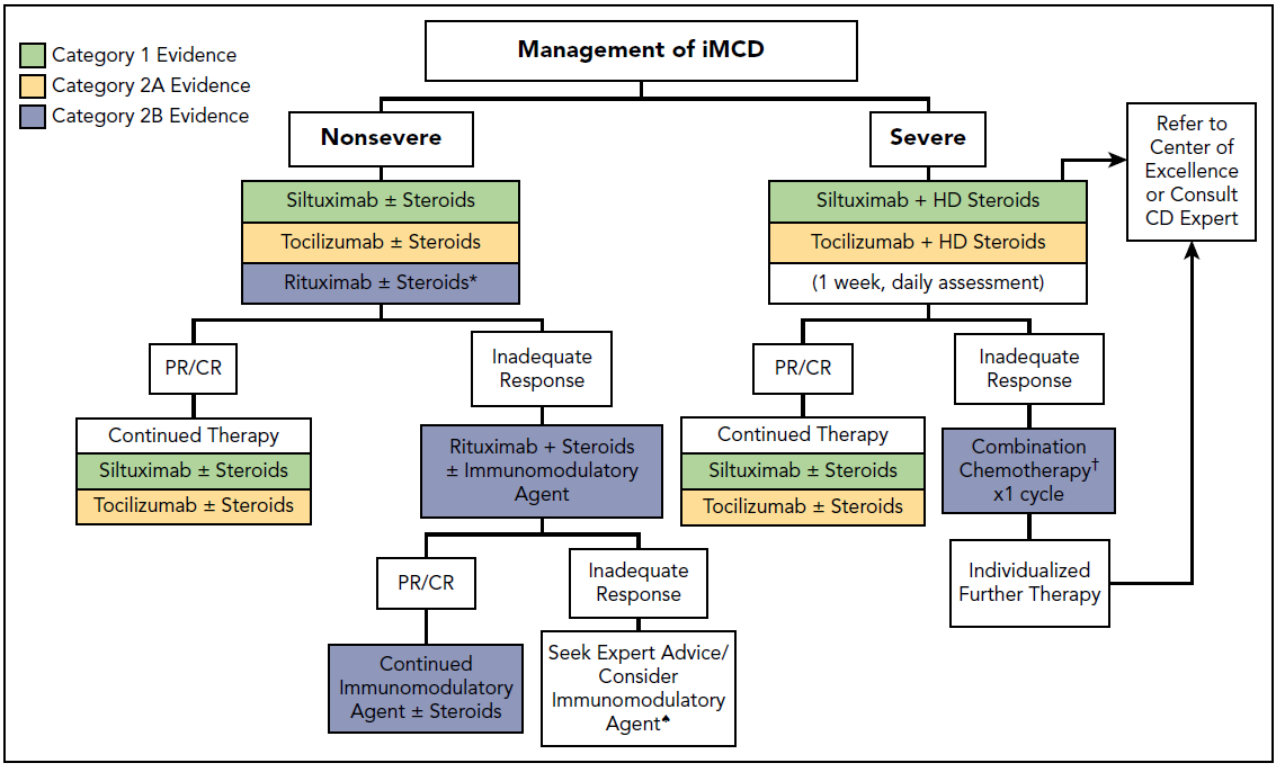

Figure 7. Management of iMCD [117]

Siltuximab is the only drug approved for iMCD by the FDA, and EMA [118–123]. It is a chimeric (human-murine) monoclonal antibody which bind and neutralize hIL-6. When hIL-6 binds its receptor the JAK/STAT pathway is activated; thus siltuximab prevent JAK/STAT signalling activation [124].

FDA's recommended dosage is of 11mg/kg every 3 weeks [118].

All histological subtypes respond to siltuximab-based therapy, thus,currently, histopathological subtype do not guide treatment. There may be a linkage between dosage received and clinical response [125].

The effects of IL-6 inhibitors on clinical symptoms are reported in Figure 8 [126].

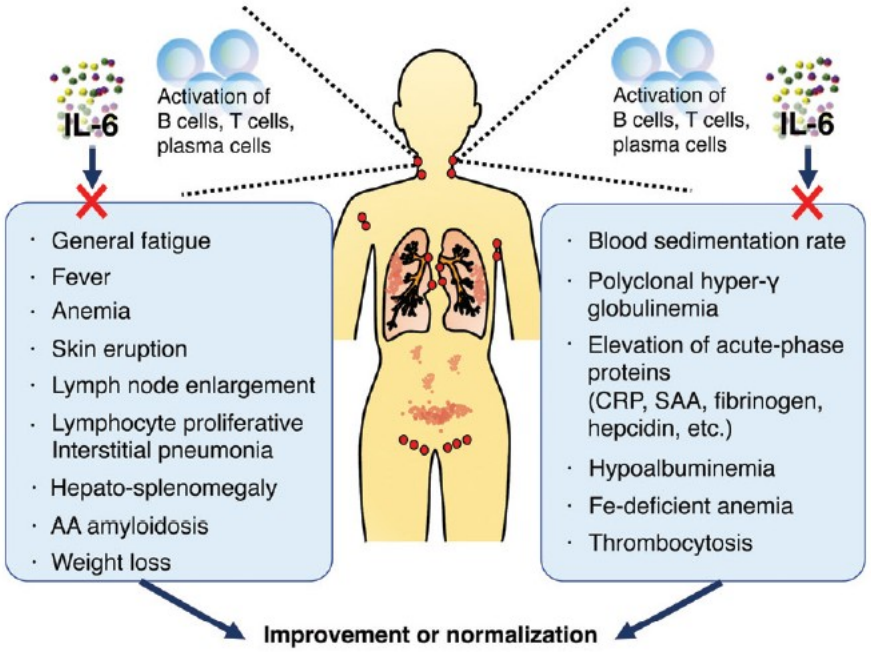

Figure 8. IL-6 inhibitors effect on clinical symptoms [126]

The only approved treatment for iMCD in Japan is Tocilizumab, a monoclonal antibody that targets both soluble and membrane-bound IL-6 receptors [62].

Others therapies that may be used with siltuximab are as follows:

- Steroids: they can suppress hypercytokinemia but since a high-dosage therpy is required it can not be made for long periods of time [125]
- Rituximab: can induce a prolonged response [125]
- Immunomodulatory agents:
  talidomide inhibit cytokine production like IL-6 and IL-1;
  bortezomib seems to reduce IL-6 levels by inhibiting kB-nuclear factor;

anakinra: IL-1 receptor antagonist;

cyclosporin: calcineurin inhibitor;

sirolimus: mTOR inhibitor [49,125,127,128]

However for patients who fail to respond to IL-6 therapy there is no clear approach. In severe refractory life-threatening iMCD, combination cytotoxic therapy using lymphoma or myeloma agents can be effective. Rituximab is used as a second-line treatment in non-severe iMCD patients. Recent reports of mTOR and JAK inhibitors in refractory-patients have generated enthusiasm. Further research is under way into mTOR, JAK/STAT and other signalling pathways and cytokines found to be increased as potential therapeutic targets [15,16].

1.13 The microenvironment in reactive lymph node

1.13.1 Lymph node architecture

Lymph node structures are located at branches of the lymphatic system. They are protected by an external fibrotic capsule with internal prolungations that form trabeculae. The cellular compartments are distributed among 3 discrete but not rigid regions: the cortex, the paracortex, and medullary cords. The cortical area is the B-cell zone containing lymphoid follicles; the paracortex contains mainly T cells and T-cell antigen presenting cells. The medullary cords contain B cells, T cells, plasma cells, macrophages, and dendritic cells [129].

Cortex

The initial cortical area contains the primary lymphoid follicles composed of naive B cells aggregates and FDCs. Antigen stimulation of these cells generates the secondary follicles composed of a mantle zone, a GC, and a dense meshwork of FDCs.

GC is a specialized lymphoid compartment in which T-cell dependent immune response occurs; furthermore, it provides a microenvironment that selects the antigen-stimulated clones that produce high affinity antibody. Antigen-selected cells then exit the GC, becoming memory B cells or plasma cells.

Two areas are present in GC: the dark zone, predominantly composed of centroblasts and the light zone, predominantly containing centrocytes and high concentration of FDCs.

FDCs are derived from mesenchymal cells and are important organizers of GC, and of T-cell dependent immune response. They express molecules that attract B and T cells facilitating the antigen-presenting process, thus they secrete CXCL13, a chemokine that recruits B and T-cells expressing CXCR5. FDCs also express CD23, ICAM-1, VCAM-1, CD21 and CD35.

Phenotypically, both centroblasts and centrocytes express mature B-cell antigens like CD20, CD19 and CD79 and GCs markers (Bcl6 and CD10). They also express surface molecules involved in cell interactions with FDCs and T cells.

GC contain specialized subpopulations of T cells important for regulating B-cell differentiation processes and T cell mediated immune response. One of these subsets are the follicular T-helper cells (TFH) mainly localized in the light zone and in the mantle area. These cells express CD4, CD57, ICOS, CXCL13, PD-1, CXCR5 and promote B cells differentiation.

GC also contain T regulatory (T-reg) cells that express CD4, CD25 and FOXP3, they seem to directly suppress B-cell immunoglobulin (Ig) production and class switch. T-reg are also found in interfollicular areas [129].

Paracortex

The paracortex is the interfollicular T cell zone, mainly containing mature T cells and interdigitating dendritic cells specialized in presenting antigens to T cells. The T cells in these areas are heterogeneous, with a predominance of CD4+ cells; some CD8+ and T-reg cells are also found.

The interdigitating cells are positive for S100, and MHC-II and negative for CD1a, CD21 and CD35.

In these areas there are also isolated large B cells with immunoblastic morphology and high endothelial venules (HEVs). Under some circumstances, plasmacytoid dendritic cells may be found in the paracortex, usually at the junction with medullary cords. These cells produce high amount of interferon α and function in the regulation of T cell responses. They express CD4, CD68, CD123, TCL1 and BDCA2 and lack specific markers of T cell, B cell, NK cell, or myeloid differentiation [129].

1.13.2 T cells

T-reg

T-reg lymphocytes are a subpopulation of CD4+ T-lymphocytes, characterized by CD25 and FOXP3 expression, that are involved in immune response suppression and self-tolerance maintenance. They exert their function in maintaining immune homeostasis by controlling immune responses and through mutual regulation between T-reg and T-effector, thus playing a relevant role in both preventing autoimmunity and facilitating tumor immune escape [130,131] .

Increased T-reg levels have been documented in both solid and lymphoid malignancies, probably contributing to tumor immune evasion by suppressing anti-tumor T-effector response [132–136]; adversely reduced T-reg levels and/or their functional impairment can result in T-effector responses against self-antigens leading to autoimmune diseases [137].

CD8+ T cells

CD8+ T cells are important against intracellular pathogens and tumors. They also contribute to regulate autoimmune disorders and allergies. Naive CD8+ T cell are activated by recognition of specific peptides presented by major histocompatibility complex (MHC) class I in peripheral lymphoid organs [138].

Tc1

Different subpopulations of CD8+ T cells exist, the best characterized is cytotoxic T cell subpopulation (Tc1) that plays a crucial role in clearance of intracellular pathogens.

Others CD8+ subpopulations include:

- Tc2 cells, they aggravate allergic diseases.
- Tc9 cells, important in anti-tumour response.
- Tc17 cells, important against viral infections, contribute to antitumor response, and have a pathognetic funtion in autoimmune diseases.

CD8+ T cells, like CD4+ T-helper cells, seems to have "lineage plasticity"; Mittrucker et al suggests that there may be a "reciprocal cross-talk" during immune response between CD4+ and CD8+ cells where not only CD4+ cells help CD8+ immune response but CD8+ cells may strongly influence the response CD4-mediated [138].

CD8+ T-reg

T-reg are essential regulator of immune tolerance and they prevent autoimmunity. Recent advances have started to reveal, in *in vitro* models, the presence of CD8+ T-reg [138].

2. Objectives

The main aim of this project is to clarify the role of the lymph node microenvironment, with particular regard to IL-6 and T-cells subsets, in the different types of CD by means of immunophenotypic and transcriptomic analysis to possibly better clarify the pathogenesis of this disease.

3. Materials and Methods

3.1. Patients selection

Patients who had a previous diagnosis of CD in between 2000 and 2022, were collected from the databases of IRCCS San Matteo Hospital Foundation of Pavia, Hospital of Varese and of Guglielmo da Saliceto Hospital of Piacenza.

All diagnostic slides were reviewed (i.e. Hematoxylin and eosin (HE) and immunohistochemical stains).

Diagnoses of CD were made according to multicentric Castleman disease (MCD) and unicentric Castleman disease (UCD) criteria [7,111].

Thirty-six patients with a CD diagnosis were initially collected and following histological revision, twenty-eight patients were therefore included in the study. We retrieved a dataset of epidemiological, pathological and clinical information including: age, sex, diagnosis, presence of B-symptoms, presence of hepatomegaly, splenomegaly, bone marrow involvement, fluid effusions, skin alterations, autoimmune diseases, POEMS syndrome, TAFRO syndrome, lymphoproliferative disorders, other diseases; laboratory data (haemoglobin, LDH, creatinine, presence of a monoclonal component, serum IL-6), virological status (HHV8, HIV, HBV, HCV, EBV, CMV), bacterial infection (tubercolosis); histopathological features, therapy, outcome and follow-up. At the end of follow-up, patients were defined as alive with/without disease, dead for complications disease-related, dead of other causes (DOC) and lost at follow up (LFU).

3.2. Pathological methods

Formalin-fixed, paraffin-embedded (FFPE) biopsies from all included cases were available for histopathological studies and stained with HE and Giemsa.

Immunohistochemical analysis with antibodies against CD20, CD79a, BCL2, CD10, BCL6, CD138, CD3, CD5, κ, λ, IgG, IgG4, Mib1/Ki-67, CD21, CD23, Cyclin D1, CD68R/PGM1, CD34, HHV8/LNA-1 (Agilent/Dako, Santa Clara, California, USA), were performed using the automated platform Dako Omnis Envision Flex.

All cases were tested for EBV presence by means of ISH, using Epstein-Barr Virus (EBER) Peptide nucleic acid (PNA) Probe linked with fluorescein for the detection of latent EBV infection on FFPE tissue sections.

3.3. T cell subset analysis

Immunohistochemical analysis with antibodies against CD4 and CD8 T-cell subsets were used to evaluate the CD4/CD8 ratio in all the affected lymph nodes. The CD4/CD8 ratio was assessed in the paracortical areas using a semiquantitative approach. Adjacent sections were immunostained respectively with antibodies against CD4 and CD8. The CD4/CD8 ratio was assessed on digital photographs of representative fields.

Antibody against FOXP3 (eBioscience/Thermofisher, Whaltam, Massachussets,USA), a specific marker of T-reg population [131], was employed to analyze this T-cell subset.

To evaluate the number of FOXP3+ cells, 10 high power fields (HPF) for each case were selected, from areas of more prominent aggregation, and the number of cells with nuclear positivity were counted under a light microscope. The mean number of FOXP3+ T-cells was calculated for each analyzed case.

For T-reg subset analysis we collected 24 non specific reactive lymph nodes from different sites (i.e. cervical, supraclavicular, axillary, abdominal, iliac, inguinal) to be used as a control group.

3.4. IL-6 assessment

Five CD cases (1 UCD, 2 iMCD, 1 HIV negative HHV8-associated MCD, 1 HIV positive HHV8-associated MCD) and a non CD reactive control were tested to assess IL-6 RNAsequence (RNAseq) expression and quantification. FFPE tissues were utilized for RNAscope and immunohistochemistry (IHC) analysis. The human IL-6 probe hybridization (Cod. 310371) was performed using RNAscope 2.5 HD Detection Reagent-BROWN (ACD, Advanced Cell Diagnostic) in accordance with the manufacturer's protocol. A dual ISH-IHC protocol was validated to simultaneously stain selected FFPE tissue slides with immunohistochemistry antibodies and RNAscope® probes.

Human tissue sections were deparaffinized, rehydrated, and unmasked using Novocastra Epitope Retrieval Solutions at pH = 6 and pH = 9 in a thermostatic bath at 98°C for 30 minutes. Subsequently, the sections were brought to room temperature and washed in PBS. After neutralization of the endogenous peroxidase with 3% H2O2 and Fc blocking by 0.4% casein in PBS (Novocastra), the sections were incubated with antibodies. For multiple-marker immunostaining, sections were subjected to sequential rounds of single-marker immunostaining, and the binding of the primary antibodies was revealed by the use of specific secondary antibodies conjugated with different enzymes. The following primary antibodies were used for IHC on human tissues: rabbit anti-human CD3 (1:100, pH = 9, Abcam), mouse anti-human CD68R/PGM1 (clone 514H12, ready to use, ph = 9, Leica Biosystems) and mouse anti-human CD31(clone JC70A, ready to use, ph = 9, Leica Biosystems).

Double IHC staining was performed by applying SignalStainBoost IHC Detection rabbit (cod. #18653, Cell Signaling Technology) alkaline phosphatase (AP)-conjugated produced in horse and Vulcan Fast Red as substrate chromogen; and SignalStainBoost IHC Detection mouse (cod. #8125S, Cell Signaling Technology) horseradish peroxidase (HRP)-conjugated produced in goat and PolyDetector HRP Green as substrate chromogen.

Slide digitalization was performed using an Aperio CS2 digital scanner (Leica Biosystems) with the ImageScope software (Aperio ImageScope version 12.3.2.8013, Leica Biosystems).

Quantitative analyses of *in situ* mRNA were performed by calculating the average percentage of positive cells in five non-overlapping GC at medium-power magnification (x200) using the HISTOQUANT software (3DHISTECH) and the output was expressed as “AREA %".

3.5. Statistical analysis

The data were described with the mean and standard deviation if continuous and with counts and percentages if categorical; they were compared between groups with the Student t test/Mann Whitney or the Fisher/$\chi 2$ test, respectively. For IL-6 analysis differences between cases were evaluated by the Kruskal-Wallis test. A two-sided P value<0.05 was considered statistically significant.

4. Results

4.1. Clinical features

The present series consists of 28 CD cases, respectively 13 UCD, 9 iMCD, 5 MCD HHV8+/ HIV-, and 1 MCD HHV8+/ HIV+. The major clinical findings are summarized in Table 8.

Table 8. Main clinical features.

Clinical and laboratory features	**All patients (*N* = 28)**			
	UCD	**iMCD**	**HHV8+ HIV- MCD**	**HHV8+ HIV+ MCD**
B-symptoms*	1/10	4/7	3/4	1/1
Anemia (Hb<13 g/dl M, <12 g/dl F)	1/10	2/7	4/4	1/1
Serum monoclonal component**	2/7	7/7	1/3	1/1
Hepatosplenomegaly	0/10	5/8	3/4	1/1
Fluid effusions	2/10	0/7	1/3	0/1
Increased creatininemia (> 1,2 mg/dl)	0/9	0/7	3/4	0/1
Proteinuria (> 15 mg/dl)	1/6	2/6	2/4	1/1
Interstitial lymphocytic pneumonia (LIP)	0/9	0/9	0/4	0/1
Peripheral neuropathy	0/13	2/9	0/4	0/1
LDH > UNL	4/10	4/6	1/4	1/1
IL-6 > UNL	2/5	3/5	1/1	1/1
HHV8 (circulating DNA and/or IHC) +	0/13	0/9	5/5	1/1
EBV serology (IgG: EBNA, VCA) +	3/4	4/6	2/2	1/1
EBV-DNA+	0/4	0/5	1/2	0/1
CMV serology (IgG) +	3/5	4/6	2/3	1/1
CMV-DNA+	0/5	0/6	2/3	0/1
HIV serology +	0/9	0/7	0/5	1/1
HCV serology +	0/9	0/7	0/5	0/1
HBV serology (anti-HBc antibodies) +	0/9	2/7	1/5	0/1

Quantiferon TB test +	0/5	0/4	0/2	0/1
Cherry hemangiomas	0/10	0/9	0/4	0/1
Secondary amyloidosis	2/13	0/9	0/5	0/1
Lymphoproliferative clonal disorder	0/13	2/9	0/4	0/1
Hemophagocytic lymphohistiocytosis	0/13	0/9	2/4	0/1
Solid tumor	1/13	2/9	2/4	1/1
Autoimmune disease***	0/13	2/9	1/4	0/1
POEMS syndrome	-	0/9	0/4	0/1
TAFRO syndrome	-	0/9	0/4	0/1

*at least one between night sweats, fever, fatigue G > 2 CTCAE, weight loss. ***monoclonal spike detected on serum protein electrophoresis and/or an abnormal (positive) serum immunofixation* ***other than Systemic Lupus Erithematosus, Rheumatoid Arthritis, Adult Still's Disease and Autoimmune Lymphoproliferative Syndrome. **Abbreviations**: Hb, hemoglobin; LDH, lactate dehydrogenase; UNL, upper normal limit; IL-6, interleukin-6; HHV8, Human herpesvirus 8; IHC, immunohistochemistry; EBV, Epstein-Barr virus; IgG, Immunoglobulin G; EBNA, Epstein-Barr Nuclear Antigen; VCA, Viral Capsid Antigen; CMV, Cytomegalovirus; HIV, Human immunodeficiency virus; HCV, Hepatitis C virus; HBV, Hepatitis B virus; HBc, Hepatitis B core antigen; TBC, Tubercolosis.

4.1.1. UCD

UCD subtype includes 13 patients, with a median age at diagnosis of 52 years (mean 44; range 6-67) and a female prevalence (9/13, 69%). Only one patient reported B symptoms (weight loss) at diagnosis; no one presented organomegaly, cherry hemangiomas or interstitial pneumonia, but 2 had a minimal fluid collection (1 pleural effusion and 1 Douglas pouch effusion).

Tests for HHV8 (respectively immunostainings and/or circulating DNA), HIV, HCV and HBV were negative in all tested cases. One patient had a previous CMV infection (documented by presence of IgG antibodies), while 2 had previous EBV infection.

AA Amyloidosis was described in 2 patients (2/13, 15%).

Serum IL6 was increased in 2/5 tested patients (range 0,3-109,1); a monoclonal component was detected in 2 cases.

Ten patients had surgical excision only, and 1 patient received corticosteroids after CD diagnosis on a lymph node biopsy. At last follow up (FU), 11 patients (84.6%) were alive without disease, while 2 were lost at follow up (LFU).

4.1.2. MCD

iMCD

iMCD subtype includes 9 patients with a median age at diagnosis of 57 years (mean 56, range 40-65) and a male prevalence (6/9, 67%). No patients had clinical symptoms related to POEMS or TAFRO syndrome, therefore they were all classified as iMCD NOS. We did not observe any case showing clinico-pathologic features consistent with the so-called iMCD-IPL (idiopathic plasmacytic lymphadenopathy) variant [4,5]. No patients had cherry hemangiomas. At diagnosis, 4/7 patients had B symptoms and 5/8 had organomegaly. HCV and HIV tests were negative in all tested cases; in 4/6 cases a previous infection by CMV (IgG positive antibodies) was documented; a previous EBV infection was detected in 4/6 patients.

Two patients had a diagnosis of autoimmune disease prior to the CD diagnosis (systemic sclerosis with limited cutaneous involvement, erythema nodosum). No IgG4-related disease was observed. Two cases of peripheral neuropathy were documented. Two patients developed a lymphoproliferative disorder during FU, respectively multiple myeloma and bone plasmocytoma (about one year after CD diagnosis in both cases).

Serum IL6 was increased in 3/5 tested patients (range 1,59-95 pg/ml); a monoclonal component was detected in all 7 tested patients.

Treatment programs were available in 5/9 iMCD cases. The patients were treated with Siltuximab (N= 3), Cyclophosphamide and steroid (N=1) or cyclophosphamide, vincristine, prednisone (CVP) (N=1); 2 patients underwent second line therapy with chemoimmunotherapy.

At last FU 5 patients (5/9, 56%) were still alive, 2 (2/9, 22%) were dead for other causes (DOC), while 2 (2/9, 22%) were LFU.

HHV8+/HIV- MCD

5 patients were analyzed, all males and with a median age at diagnosis of 75 years (mean 69, range 35-83). One case had a previous history of HIV- Kaposi sarcoma. B symptoms and hepatosplenomegaly at diagnosis, were observed in 3 cases. Hemophagocytic lymphohistiocytosis was reported in 2 patients.

HCV tests were negative in all tested cases (0/5) but 1 patient had immunity for HBV. One patient had active CMV infection, one a viral reactivation and another one a previous infection.

Serum IL-6 value was available in a single case only, and it was increased (67,76 pg/ml).

Treatment schedules were available in 2 cases: one underwent rituximab single agent therapy, the other received liposomal doxorubicin as first line therapy and paclitaxel and etoposide as second and third line respectively because of concurrent Kaposi sarcoma.

At last FU 1 patient (1/5, 20%) was DOC, 2 (2/5, 40%) were dead for complication disease related (i.e. hemophagocytic lymphohistiocytosis) while 2 (2/5, 40%) were LFU.

HHV8+/HIV+ MCD

A 54 year old patient presented at diagnosis with hepatosplenomegaly and B symptoms. He had a previous history of HIV+ Kaposi sarcoma. HCV and HBV tests were negative but he had previous CMV and EBV infections.

Serum IL6 was increased (44,4 pg/ml) and a monoclonal component was detected.

Treatment regimen included liposomal doxorubicin and rituximab, at last FU the patient was still alive.

4.2.1. Histopathological features

46% of patients (13/28) had HV (Figure 9) or HyperV type histology (11/13 UCD, 2/15 MCD); 11% (3/28) had a PC type (3/15 MCD) (Figure 10) and 43% (12/28) had a mixed type (2/13 UCD; 10/15 MCD). In one patient (iMCD with mixed histology) CD changes were detected both in lymph node and in the lymphoid cellular infiltrate involving the subcutis of the left thigh; bone marrow CD localization was found in a single case (HHV8+/HIV- MCD with mixed type histology).

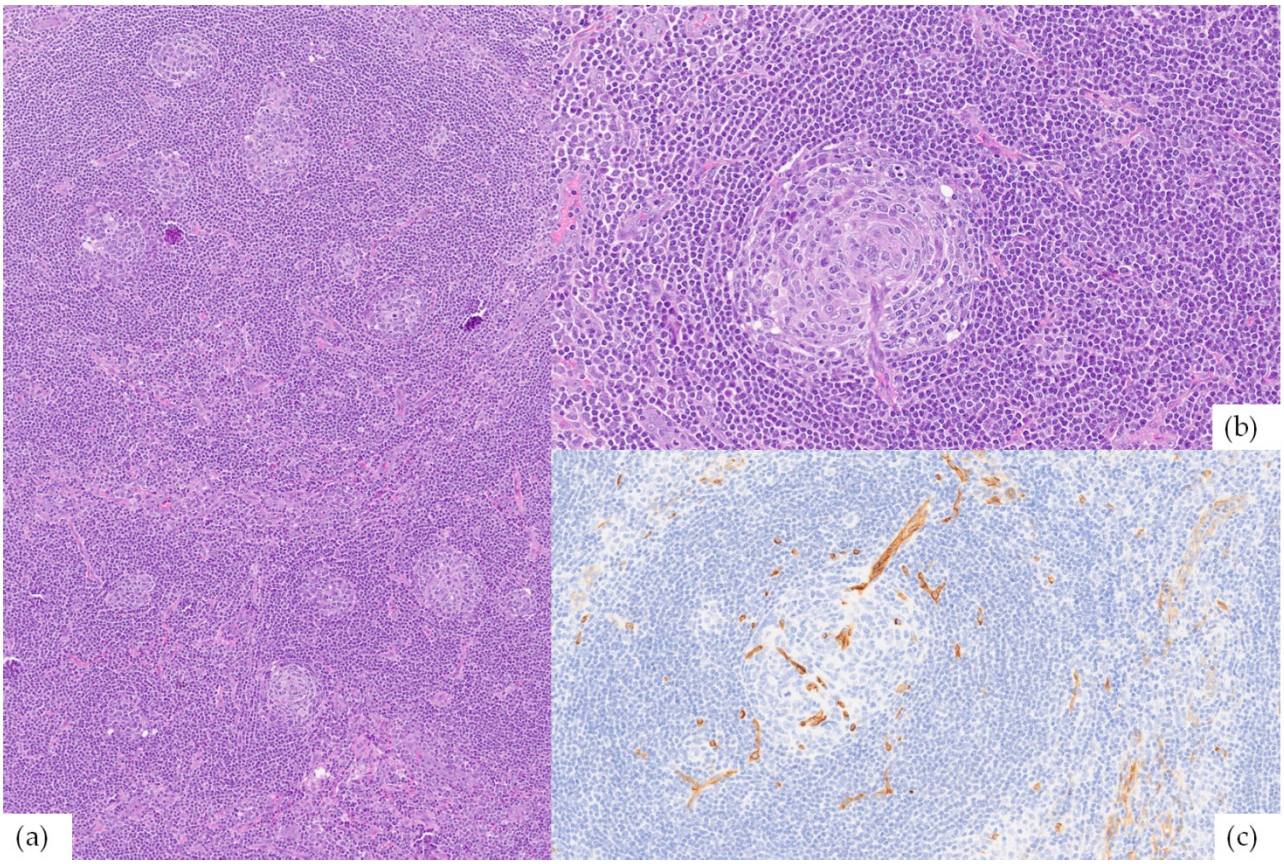

Figure 9. Hyaline vascular histology shows multiple regressed germinal centers within the same, expanded mantle zone ("twinning")(a, hematoxylin and eosin, 10x) and increased vascularity with radially penetrating vessels ("lollipop") (b, hematoxylin and eosin, 20x; c, CD34 immunostaining, 20x).

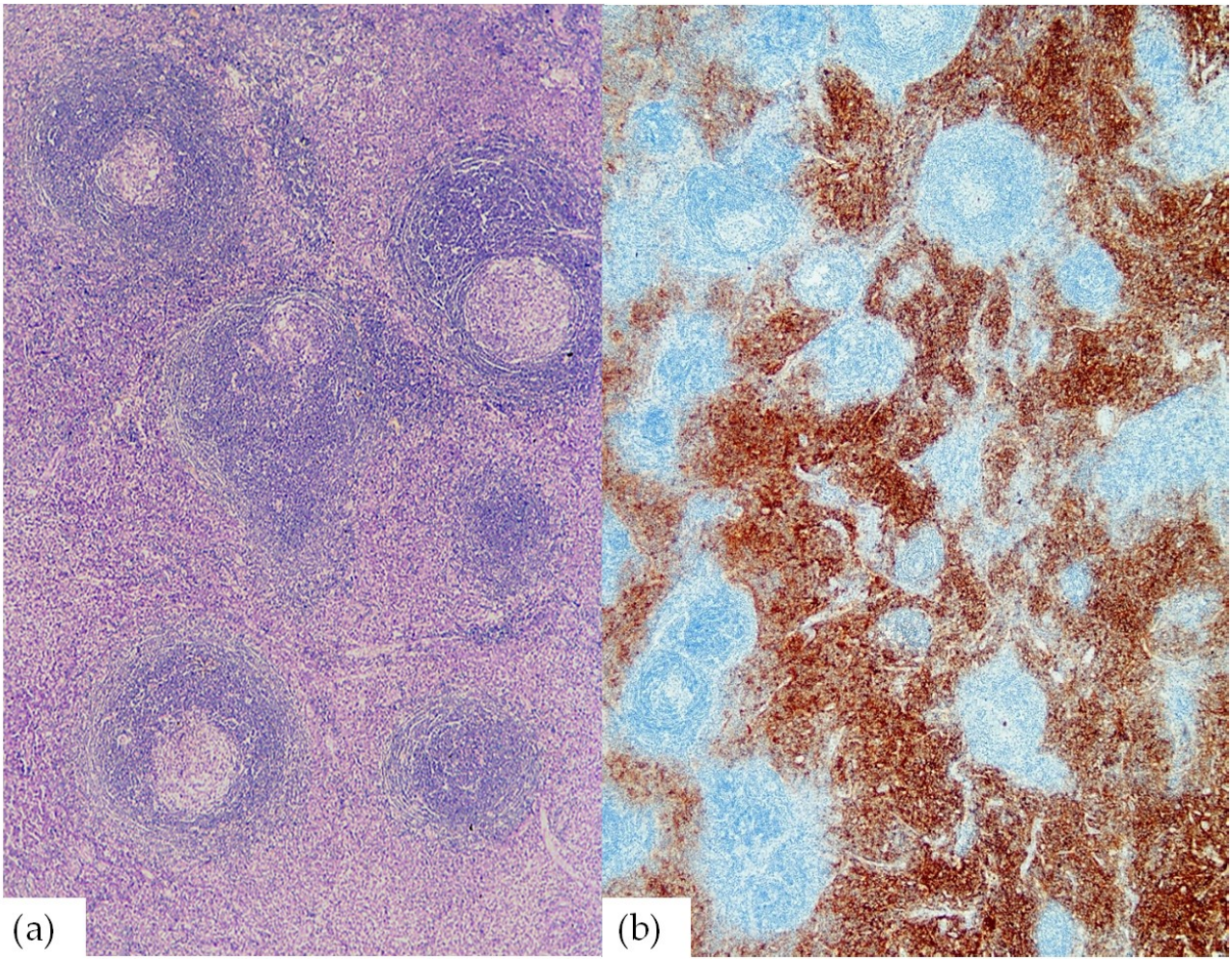

Figure 10. Plasma cell histology shows variably sized, mostly hyperplastic, germinal centers (usually hyperplastic) (a, hematoxylin and eosin, 10x) and sheet-like plasmacytosis (b, CD138, 10x).

Follicle B centers with hyperplastic/dysplastic follicular dendritic cells were found in 5/28 (18%) cases (3 HV-UCD, 1 mixed type MCD, 1 PC-MCD).

In most cases (26/28, 2 cases could not be evaluated due to technical issues) the plasma cells population showed polytypic Ig light chains expression by IHC; 3/28 had an IgG4/IgG > 40% and IgG4 > 10/HPF, but did not meet the diagnostic criteria for IgG4-RD [94,95,139].

EBV was negative in all cases (28/28).

4.2.2. T-cell subset analysis

CD4/CD8 analysis

The CD4/CD8 ratio was decreased in 9 cases: 1 case (HHV8+HIV+MCD mixed) showed a predominance of CD8+ T-cells, 4 cases showed a similar amount of CD4+ and CD8+ T-cells (3 HV-UCD, 1 UCD mixed type), 4 cases had still a predominance of CD4+ T-cells but with a significant increase in the CD8+ population (1 HV-UCD, 1 UCD mixed type, 1PC- iMCD, 1 HHV8+ MCD mixed type) (Figure 11).

No statistically significant differences in terms of CD4/CD8 ratio changes were found when comparing different clinical (UCD versus MCD (as a whole), p=0,2275; UCD versus iMCD versus HHV8+MCD, p= 0,2232), histological (CD HV/HyperV versus CD mixed versus CD PC, p=0,9896) and clinico-pathological subtypes of CD (HV-UCD versus mixed UCD versus HyperV-MCD versus mixed MCD versus PC-MCD, p= 0,2039) (Figure 12).

Architectural assessment of T-cell subpopulations revealed that both CD4+ and CD8+ T-cells were distributed in their specific nodal paracortical/interfollicular compartments; CD4+ T cells being more numerous around the GC and CD8+ lymphocytes beyond.

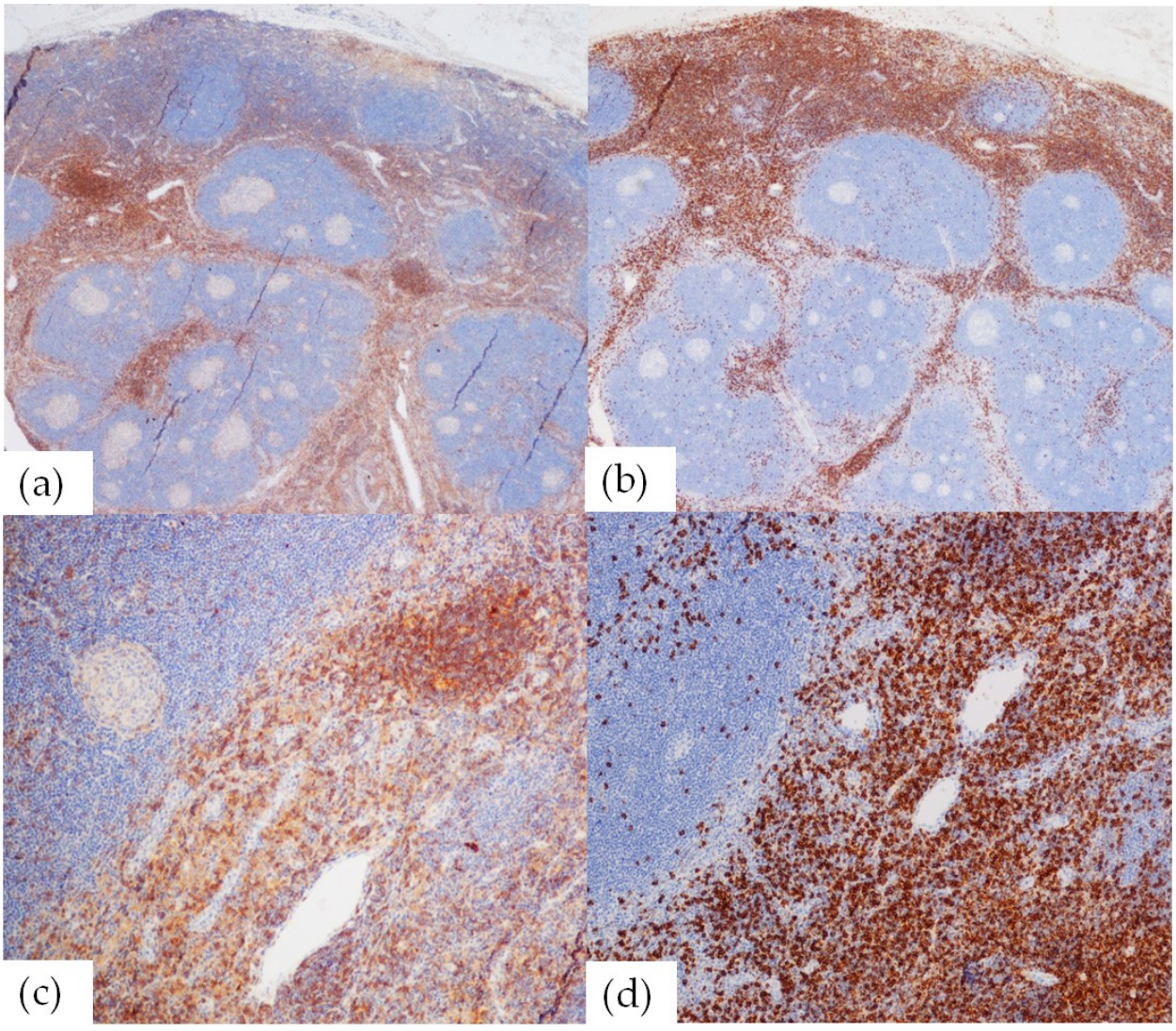

Figure 11. Nodal immunostainings show a CD4/CD8 ratio in favor of CD8+ T-cell population noticeable at both low a) CD4+ T-cells x2; b) CD8+ T-cells x2, and higher magnification c) CD4+ T-cells 10x; d) CD8+ T-cells x10.

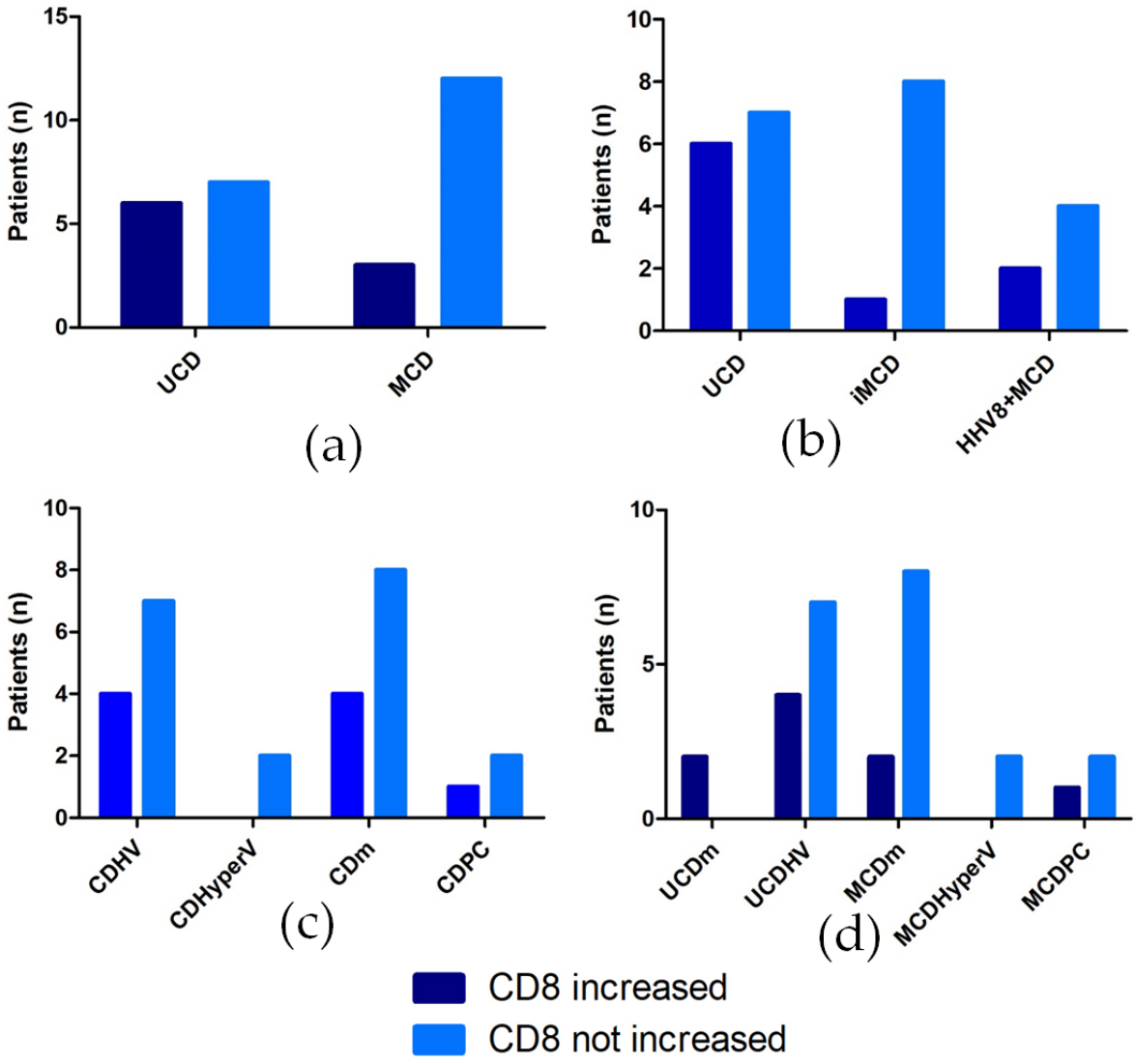

Figure 12. Evaluation of CD4/CD8 ratio in different CD subtypes. a) Evaluation of decreased CD4/CD8 ratio versus normal CD4/CD8 ratio in UCD and MCD cases; b) Evaluation of decreased CD4/CD8 ratio versus normal CD4/CD8 in UCD versus iMCD versus HHV8+ MCD; c) Evaluation of decreased CD4/CD8 ratio versus normal CD4/CD8 in the various histological subtypes; d) Evaluation of decreased CD4/CD8 ratio versus normal CD4/CD8 in the various clinico-histological subtypes. Dark blue bars indicate cases with decreased CD4/CD8 ratio (increased quantity of CD8+ cells), light blue bars indicate cases with normal CD4/CD8 ratio. **Abbreviations:** UCD, unicentric Castleman disease; MCD, multicentric Castleman disease; iMCD, idiopathic multicentric Castleman disease; UCDm, unicentric Castleman disease mixed type; UCDHV, unicentric Castleman disease hyaline-vascular type; MCDm, multicentric Castleman disease mixed type; MCDHyperV, multicentric Castleman disease hypervascular type; MCDPC, multicentric Castleman disease plasma cell type.

T-reg analysis

Immunohistochemical staining for FOXP3+ T-reg was carried out on 28 CD and 23 control samples (reactive lymph nodes). The number of FOXP3+ T-cells in CD affected lymph nodes was significantly ($p<0,0001$) lower (mean 23,18±20,18) in comparison to the number in the control samples (mean 58,63±28,04). In UCD the numbers of FOXP3+ T-reg (mean 26,54±26,87) was compared, respectively to the numbers of T-reg in MCD cases as a whole (mean 20,27±12,11), in iMCD (mean 17,33±12,04) and in HHV8+ MCD (mean 24,67±11,83). However the statistical analysis of T-reg distribution in these different CD clinical subtypes did not reveal any statistically significant differences ($p= 0,9083$; $p= 0,5965$) among the different CD forms.

Similarly, we compared the FOXP3+ T-reg numbers in the different CD histologic subtypes, respectively HV/HyperV (mean 27,23±26,61), PC (mean 22,33±4,509) and mixed (mean 19±13,59) but no statistically significant differences ($p=0,8167$) emerged. Subsequently, we compared the number of FOXP3+T-reg with respect to the different clinico-pathological CD subtypes, including respectively UCD HV (mean 27±29,07), UCD mixed (mean 24±14,14), MCD HyperV (mean 28,5±6,364), MCD mixed (mean 18±14,03), MCD PC (mean 22,33±4,509); in this analysis, no statistically significant differences ($p= 0,8205$) were found.

The comparison between the number of FOXP3+ T-reg in cases with an increase in the CD8+ T-cell population (mean 28,56±17,04) and cases without CD8+ T-cell subset increase (mean 20,63±21,45), revealed no statistically significant difference ($p=0,0991$) (Figure 13)

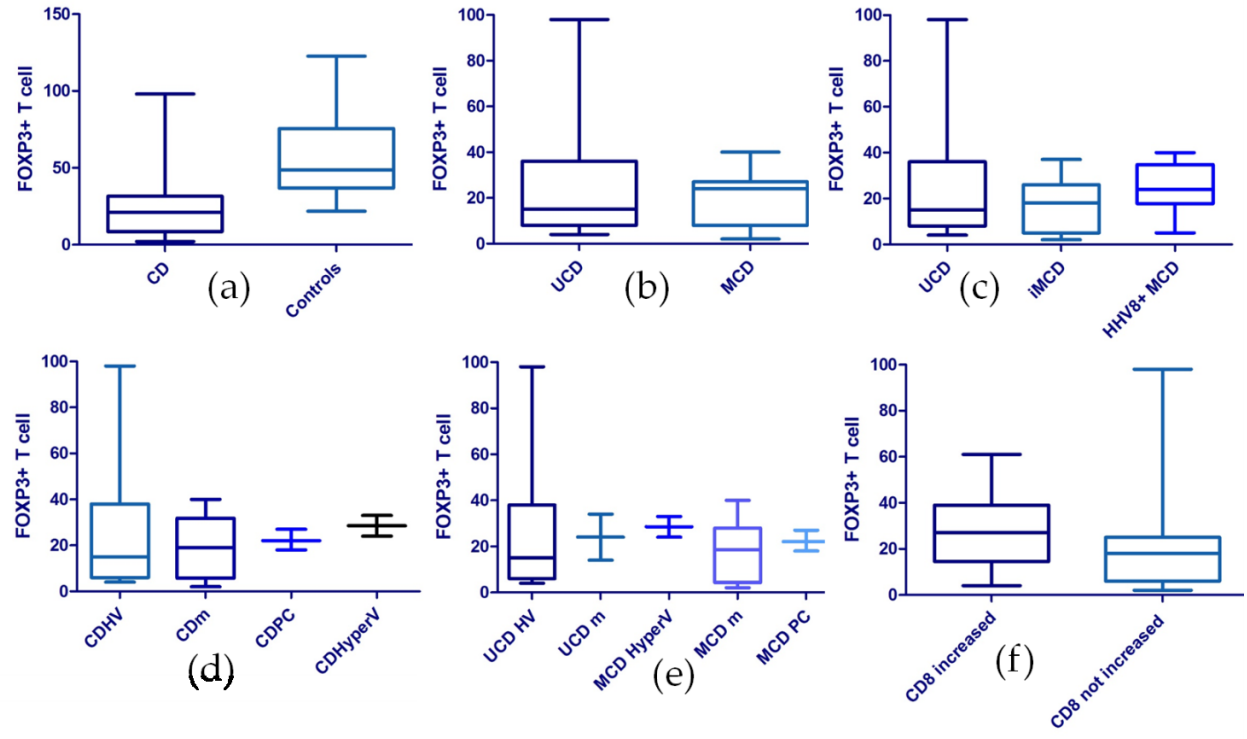

Figure 13. FOXP3 analysis. a) FOXP3 analysis in CD patients versus controls shows a statistically significant ($p<0,0001$) difference between the 2 groups; comparison of FOXP3+ cells instead did not show statistically significant differences between the various CD clinical subtypes: b) UCD versus MCD; c) UCD versus iMCD versus HHV8+ MCD. Likewise statistically significant differences were not found comparing d) the various pathological subtypes or e) the different CD clinico-pathological subtypes nor f) comparing cases with an increased CD8+population versus cases without. **Abbreviations:** CD, Castleman disease; UCD, unicentric Castleman disease; MCD, multicentric Castleman disease; iMCD, idiopathic multicentric Castleman disease; HV, hyaline-vascular; HyperV, hypervascular; m, mixed type; PC, plasma-cell type.

4.2.3 IL-6 assesment

IL-6 RNAseq could be assessed in 5 CD patients (1 UCD, 2 iMCD, 1 HHV8+HIV- MCD, 1 HHV8+HIV+ MCD) (see Table 9 for the main clinical and histological features) and a non CD reactive control.

Table 9. Main clinical and histological features.

N	Age	Sex	UCD vs MCD	Histology	CD8 increased	FOXP3	sieric IL-6	HHV-8	HIV	Therapy	Outcome
3	75	M	MCD	CD mixed	no	24	67,7	+	-	Rituximab	Dead for complication disease related
6	43	M	UCD	CD HV	yes	61	6,02	-	-	Surgery	Alive
14	57	M	MCD	CD HyperV	no	24		-	-		LFU
22	54	M	MCD	CD mixed	yes	24	42,7	+	+	Doxorubicin +Rituximab	Alive
23	56	F	MCD	CD mixed	no	37	24,7	-	-	Siltuximab	Alive

Abbreviations: UCD, unicentric Castleman disease; MCD, multicentric Castleman disease; CDHV, Castleman disease hyaline-vascular type; CDHyperV, multicentric Castleman disease hypervascular type; LFU, lost at follow up.

RNA ISH, which is more sensitive and specific than IHC, was used to identify the lymph node structures and cells expressing IL-6. All tested cases showed IL-6 expression in the lymph node (Figure 14) with a higher expression in CD cases compared to the control.

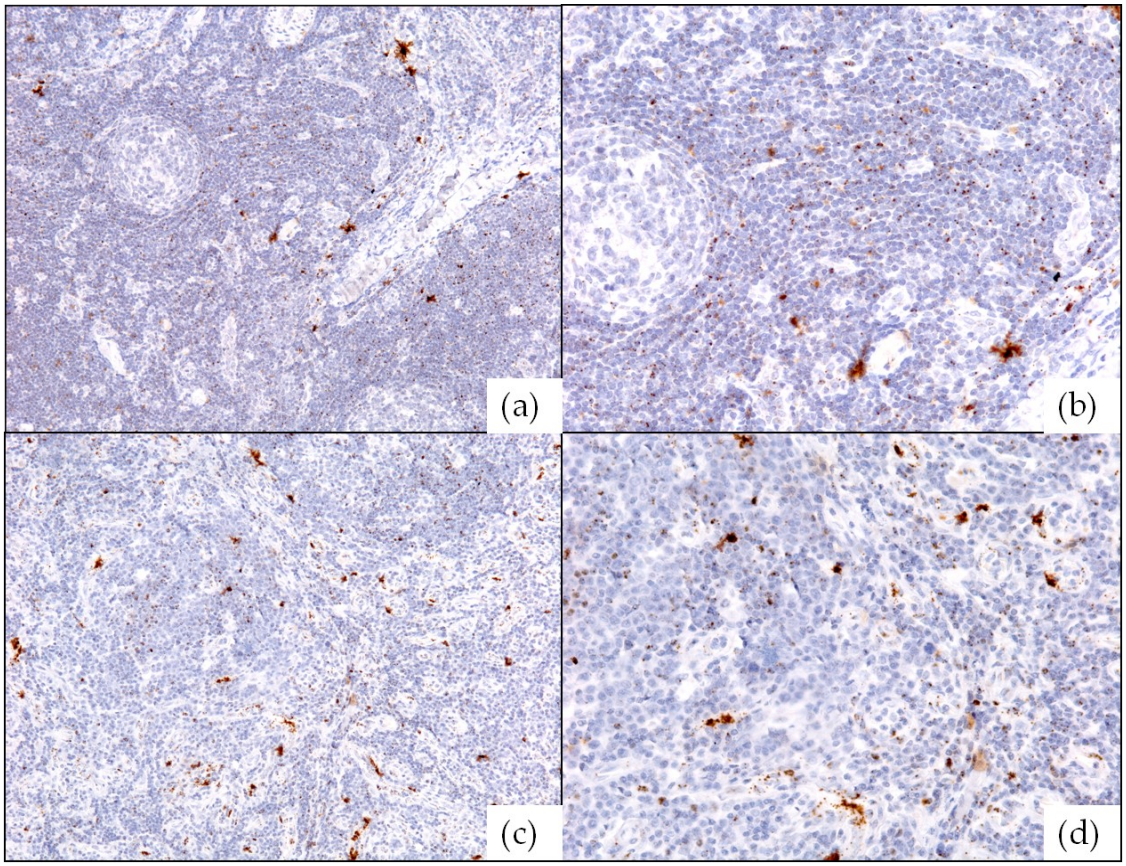

Figure 14. IL-6 expression in one of the iMCD cases with HyperV histology at lower (a), and higher magnification (b); and in HHV8+HIV- MCD case with mixed histology (c;d).

Particularly, quantification of IL-6 RNAseq was held on 5 fields at 20x magnification (Table 10)

Table 10. IL-6 expression assessment.

IL-6 expression % area	HV-UCD	iMCD HyperV	iMCD mixed	HHV8+HIV- MCD mixed	HHV8+HIV+ MCD mixed	Control
	0,62	0,42	0,29	0,77	0,77	0,13
	0,21	0,2	0,69	0,47	0,77	0,14
	0,27	0,22	0,75	0,65	1,15	0,06
	0,3	0,25	0,37	0,5	0,57	0,16
	0,34	0,31	0,34	0,32	1,04	0,08
median	0,3	0,25	0,37	0,5	0,77	0,13
mean	0,35	0,28	0,49	0,54	0,86	0,11

Abbreviations: HV-UCD, unicentric Castleman disease hyaline-vascular type; iMCD, idiopathic multicentric Castleman disease; HyperV, hypervascular type.

Were also conducted post-hoc analyses that showed statistically significant differences (p=0.0005) in IL-6 expression between HHV8 positive cases and control (Figure 15).

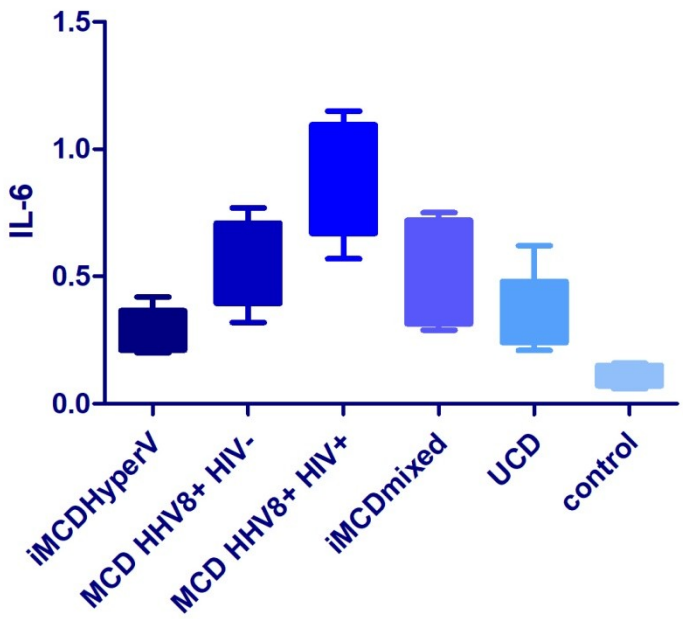

Figure 15. Analysis of IL-6 expression in the 5 cases and in the control. **Abbreviations:** iMCD, idiopathic multicentric Castleman disease; HyperV, hypervascular type;MCD HHV8+ HIV-,HIV negative HHV8-associated multicentric Castleman disease; MCD HHV8+ HIV+, HIV positive HHV8-associated multicentric Castleman disease; UCD, unicentric Castleman disease.

In 4 cases sieric IL-6 was available, and was confronted with the median IL-6 RNAseq expression in lymph node (Table 11) showing that HHV8+ cases had a higher value of both sieric IL-6 and lymph nodal IL-6 RNAseq.

Table 11. Sieric and IL-6 RNAseq expression in CD cases

	HV-UCD	iMCD HyperV	iMCD mixed	HHV8+HIV- MCD mixed	HHV8+HIV+ MCD mixed
sieric IL-6	6,02		24,7	67,5	42,7
median IL-6 RNAseq in lymph node	0,3	0,25	0,37	0,5	0,77

Abbreviations: HV-UCD, hyaline-vascular unicentric Castleman disease**,** iMCD, idiopathic multicentric Castleman disease; HyperV, hypervascular type;MCD HHV8+ HIV-,HIV negative HHV8-associated multicentric Castleman disease; MCD HHV8+ HIV+, HIV positive HHV8-associated multicentric Castleman disease.

The histomorphological pattern of IL-6 RNA ISH was noted in scattered dispersed cells mostly in the interfollicular areas, thus we conducted dual RNAscope/IHC staining to find the cellular source of the elevated IL-6 expression.

The dual staining did not reveal significant co-expression of IL-6 and CD3 or CD68/PGM1, instead, at histomorphological evaluation, the IL-6 expressing cells tracked along hypervascular areas in the interfollicular areas (Figure 16)

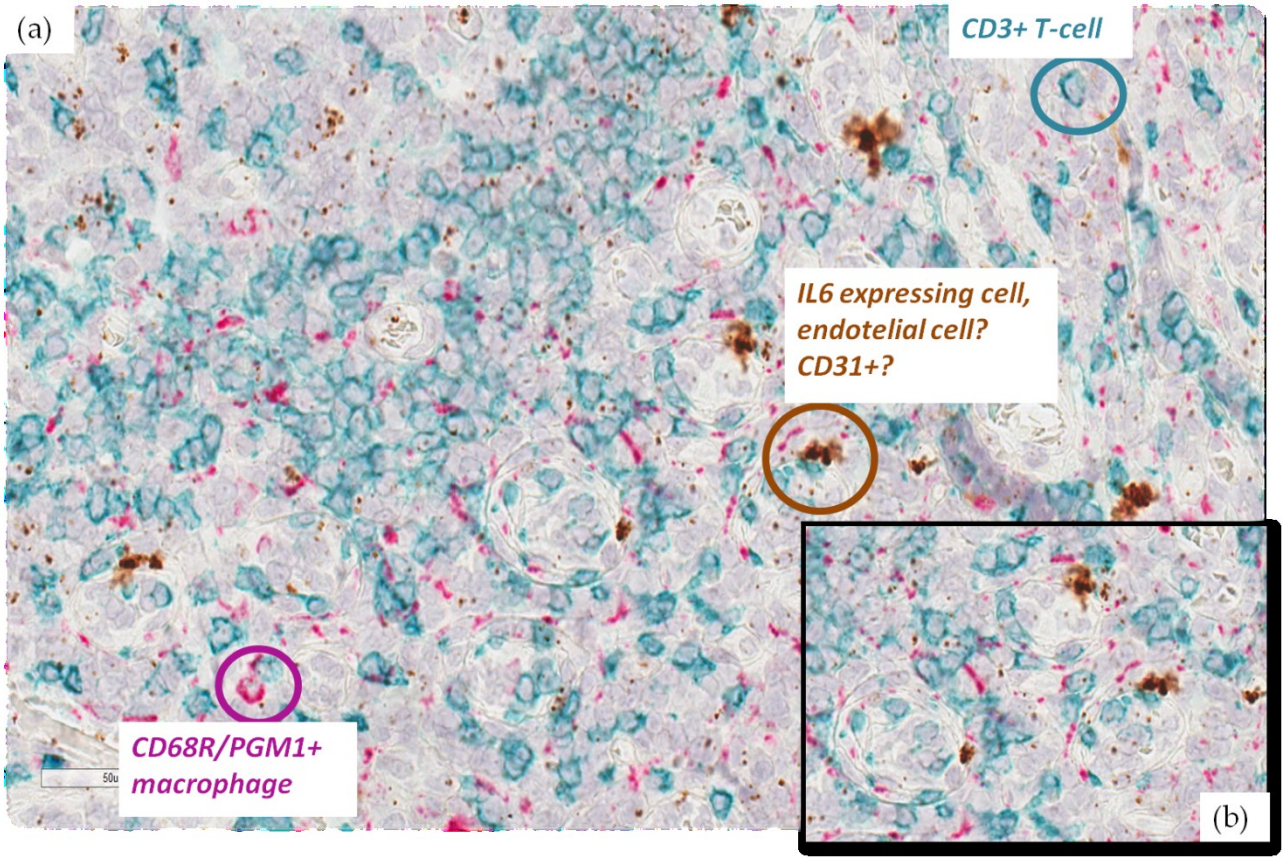

Figure 16. Dual RNAscope/IHC showing IL-6 expressing cells (brown), CD3+T-cells (blue) and CD68R/PGM1+ hystiocite-macrophages (red) (a); higher magnification of IL-6 expressing cells (b)

So, dual RNAscope (IL-6)/IHC (CD31) staining were conducted that showed that IL-6 was overexpressed in CD31-positive endothelial or lymphatic cells in all of the CD tested cases, thus suggesting that vasculature-associated cells are the major source of IL-6 in CD (Figure 17). The dual staining instead did not show co-expression of IL-6 and CD31+ cells in the control case (Figure 18).

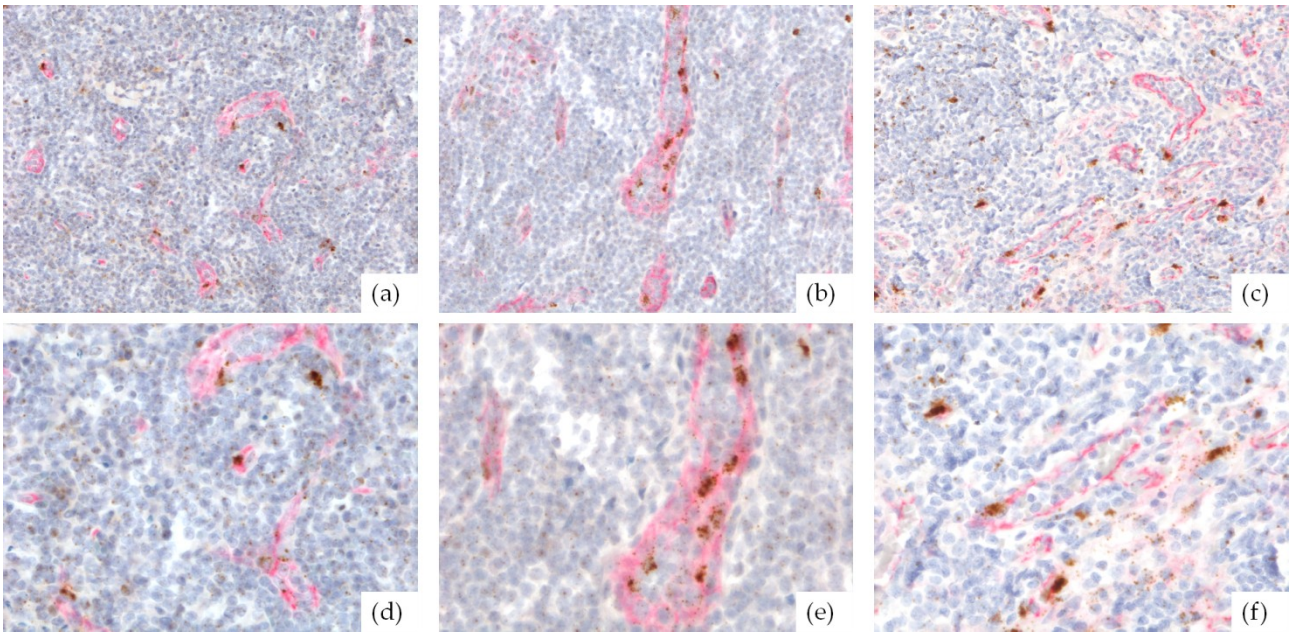

Figure 17. Dual RNAscope/IHC showing that CD31+cells (red) are the major source of IL-6 (brown) in CD; UCD HV (a); iMCD mixed (b); HHV8+HIV+MCD mixed (c); higher magnification of UCD HV(d), iMCD mixed (e), HHV8+HIV+MCD mixed (f).

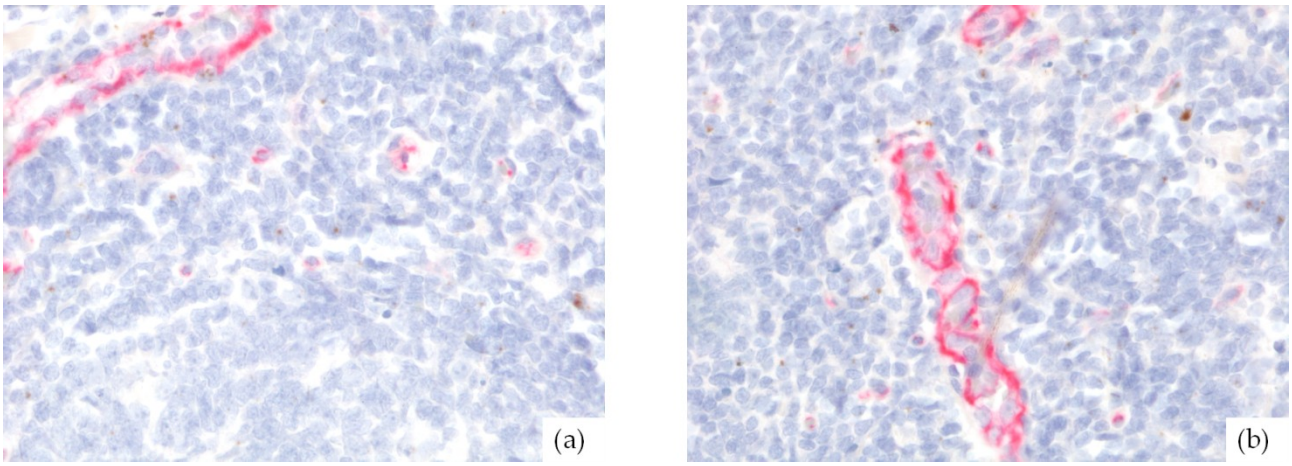

Figure 18. Dual RNAscope/IHC showing IL-6 expressing cells (brown) and CD31+ cells (red) in the control case at lower (a) and higher magnification (b).

5. Discussion

Several studies contributed to highlight the major biological features of CD, mainly including the central pathogenetic role of IL-6 and other inflammatory cytokines [9,14,15,46,62]. Furthermore, data accumulated pointing out the crucial role of local (nodal) microenvironment among the major sources of hypercytokinemia [28,29]. With this respect, it seems reasonable that the interplay between the T-cells population and other components of the local (nodal) microenvironment might play a crucial role for defining the inflammatory *milieu* and the various modalities of immune response.

Nevertheless, only few previous studies focused on the nodal cellular composition and distribution of accompanying T cell subsets and on the analysis of lymph node trascriptome in the different CD subtypes [28,29,49,140].

The aim of this study is to contribute to elucidate the immunophenotypic features and distribution of the T cellular background and to assess the expression and quantification of IL-6 RNAseq by trascriptomic analysis in nodal biopsies within the different subtypes of CD.

The CD4+/CD8+ T cells ratio assessment documented an increase in CD8+ T-cells in 32% of CD patients. Such increase occurred irrespectively of clinico-pathological subtype of CD but it was more frequently associated with the UCD (6/13 cases, 46%, 4 of them exhibiting HV histology and 2 mixed histology).

In the others CD subtypes, the CD8+ T lymphocytes nodal tissue levels were increased in 1/9 (11%) iMCD, 1/5 (20%) HHV8+ HIV- MCD, and in the single case of HHV8+ HIV+ MCD. As to the histopathologic features of the MCD cases with altered CD4/CD8 ratio, the single case of iMCD had a PC histology, whereas both HHV8+ cases showed a mixed histology.

Fajenbaum et al. reported by means of flow cytometry analysis an increased level of CD8+ T-cells in the peripheral blood of patients with iMCD [141] and iMCD-TAFRO, during disease flares [49]. Furthermore, in the iMCD-TAFRO study the proportions of CD4+ and CD8+ T-cells were also assessed in lymph node tissue always by flow cytometry, showing in both analyzed cases an increased proportion of CD8+ T-cells [49].

The data of this study document that increased CD8+ T cell levels may occur also in others CD forms including UCD and HHV8+ MCD, suggesting that imbalances in CD4/CD8 ratio may be shared by different CD subtypes.

Studies about the neoplastic microenvironment in murine models showed that IL-6 might mobilize a T-cell antineoplastic immune response, promoting differentiation of naive CD8+ T cells in specific CD8+ effectors [142–144]. On such findings, increased IL-6 levels in CD could similarly favour the expansion of CD8+ T cell subpopulations. The limited data on IL-6 values in this series prevent from any significative conclusions, nevertheless the 3 patients with a decreased CD4/CD8 ratio with availability of serum IL-6 values, also showed serum IL-6 values higher than normal.

As at least a subset of CD might be associated to disruption of immune system control I tried to assess the role of a peculiar subset of regulatory T-cells, known as T-reg that can be identified by means of specific immunophenotypic markers. T-reg lymphocytes are a subpopulation of CD4+ T-lymphocytes, that are involved in immune response suppression and self-tolerance maintenance [130,131].Various studies documented increased T-reg levels in both solid and lymphoid malignancies, probably contributing to tumor immune evasion by suppressing anti-tumor T-effector response [132–136]; adversely reduced T-reg levels

and/or their functional impairment can result in T-effector responses against self-antigens leading to autoimmune diseases [137].

On such bases, the number of FOXP3+ T-reg cells were assessed in the CD series as well as in controls (non specific reactive lymph nodes). Immunostainings revealed a lower mean number of FOXP3+ T-reg cells in CD cases, when compared with controls (23,18 in CD vs 58,63 in reactive lymph nodes). This difference in FOXP3+ T-reg cells nodal tissue levels was statistically significant by means of Mann Whitney test ($p<0,0001$). In contrast, no statistically significant difference in the number of T-reg lymphocytes was found among the different CD clinico-pathological subsets. No statistically significant correlation between the FOXP3+ T-reg count and the CD4/CD8 ratio were found. The decreased number of FOXP3+ T-reg lymphocytes observed suggests that pathogenetic mechanisms similar to those that can lead to autoimmune diseases might be involved also in CD. Conversely, a few studies documented an increased level of intratumoral T-reg cells in patients with B-cell lymphomas, leading in some cases to inhibition of intralesional CD8+ T-cells [132,133]. Based on these findings, it may be hypothesized that CD develops through different pathways than most B-cell lymphomas.

In literature, only two recent studies focusing on CD's trascriptome and cytokine microenvironment have been published. Moreover, they provided contrasting results about IL-6 increased expression in the lymph node because Wing et al found an increased expression of IL-6 in MCD but this was not confirmed in the study by Horna et al [28,29].

On such bases, RNA ISH was used to assess IL-6 expression and, if present, its histomorphological pattern in 5 lymph nodes with different clinico-pathological subtype of CD (HV-UCD, HyperV-iMCD, iMCD-mixed, HHV8+/HIV+ MCD mixed, HHV8+/HIV- MCD mixed) and in a non CD reactive control. IL-6 was found to be increased in all of the analyzed CD cases, in contrast with the study of Wing et al where IL-6 expression was increased only in MCD cases [28].

The median IL-6 expression value was higher in HHV8+ cases and that may be related to the presence of vIL-6 that stimulates secretion of hIL-6 with a paracrine fashion since vIL-6 upregulate VEGF, similar to hIL-6, and VEGF further induces hIL-6 expression from endothelial cells [15]. This is also in line with the finding that IL-6 tracked along CD31 expressing vascular structures that could be of endothelial or lymphatic origin. IL-6 expression by CD31+ cells was noted in all the CD analyzed cases, not only in HHV8+ ones, suggesting that vasculature-associated cells may be a significant source of IL-6 in CD. CD31+ cells expressing IL-6 were also noticed in the study by Wing et al, further supporting this hypothesis.

Lastly, as mentioned above, IL-6 might mobilize a T-cell antineoplastic immune response, promoting differentiation of naive CD8+ T cells in specific CD8+ effectors [142–144], so increased IL-6 levels in CD could favour the expansion of specific CD8+ T cell subpopulations. In the 5 CD cases analyzed for IL-6 lymph nodal expression, 2 of them (HV-UCD and HHV8+/HIV+ MCD) showed increased expression of CD8+T cells, however the very limited number of cases prevent from any conclusion.

In conclusion, this study has documented that in all the different clinico-pathological subtypes of CD, changes may occur in the composition of nodal CD T-cell background, particularly including a frequent decrease of CD4/CD8 ratio and a reduction of FOXP3+ T-reg cells. Furthermore, the findings about IL-6 expression seem to suggest that despite CD heterogeneity some similarities may exist in the lymphonode microenvironment of different CD types. In this respect, this analysis seems to indicate that the major source of IL-6 production may be CD31+ endothelial cells.

6. Conclusions

CD is a rare disease that identifies a group of heterogeneous lymphoproliferative disorders, thus consisting of several subtypes with different etiologies, clinical manifestations, and histological features. All these features make account for the difficulties to collect data from large series and to compare the different CD subtypes.

The data collected in this study documented that changes may occur in the composition of nodal CD T-cell background and, according to IL-6 RNAseq results, that despite CD heterogeneity some similarities may exist in the lymphonode microenvironment of different CD types.

Further studies will be held to better elucidate these findings, through mass spectroscopy analysis, to show if IL-6 higher values that have been found in CD lymph node are also confirmed by proteomic analysis. This analysis will be done through laser microdissection of the vessels' containing areas also to verify that endothelial cells are the major source of IL-6 in the local microenvironment.

Lastly, other studies will be focused on plasmacytoid dendritic cells (pDCs) since foci of hyperplastic pDCs are found in the lymphnode interfollicular areas, near endothelial cells; pDCs are an important connection between innate and adaptative immune response, they are significant against intracellular pathogen, and crucial to help modulating T-cells response. Thus, to evaluate a possible role of pDCs in CD and their possible relationship with endothelial cells a trascriptomic analysis by digital spatial profiling will be done, and possibly unravel unknown pathogenetic features that may also be helpful to discover new therapeutic targets.

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