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MED 08

**Dissecting the microenvironment of Splenic Marginal Zone  
Lymphoma and Diffuse Large B cell Lymphoma to find new stromal  
and immunological predictive biomarkers and targets.**

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## INTRODUCTION

For many years cancer biology has been focused on the understanding the genetic alterations harbored by neoplastic cells that distinguish these cells from their normal counterparts. Multiple genetic and epigenetic changes promote the initiation of tumorigenesis and allow growth and survival.<sup>(1-2)</sup>

More recently, the role of the tumor microenvironment (TME) has gained a increasingly interest for its recognition as a key factor in multiple stages of tumorigenesis and neoplastic progression, as well as in local resistance, immune-escaping, and distant metastasis both in solid and lymphoid neoplasms.<sup>(3)</sup> Numerous evidences indicate that tumour-associated immunological and stromal microenvironment play a critical role in the subsequent development of more advanced and refractory malignancies.<sup>(4)</sup>

Lymphomas represent an ideal setting to dissecting the tumor microenvironment because these neoplasms arise from cells that depend on numerous highly concerted interactions with immune and stromal cells in the course of normal development.

The present research is aimed on evaluating the tumour-associated immunological and stromal microenvironment in B-cell malignancies. In particular, the reciprocal influence of immune cells and stromal elements on the development and progression of Splenic Marginal Zone Lymphoma (SMZL) has been investigated, where a deregulated immunologic stimulation may have a pathogenetic role. The microenvironment of the bone marrow (BM) represents an elective disease localization endorsing diagnostic and prognostic relevance.

The main goal was to identify new tools for recognition of early stage of progression disease, potential prognostic markers and therapeutic targets, by means the examination of the tumor microenvironment, which seems to be a key factor in multiple stages of disease progression and a pathological active niche for tumor evolution.

## **THE HAEMOPOIETIC SYSTEM**

The haemopoietic system consists of bone marrow, thymus, spleen, lymph nodes and lymphatics (the small vessels that link the lymph nodes and returns excess fluid to the circulation). It plays a role in the defence against infection and cancer and lymphoid organs that are part of it may be classified in primary and secondary ones. The primary lymph organs are the bone marrow and the thymus, where lymphocytes are generated. In the secondary lymph organs mature and naïve lymphocytes are maintained to initiate adaptive immune responses. Secondary lymph organs include the lymph nodes, the spleen and the mucosal lymphoid tissue. <sup>(5)</sup>

The lymph nodes are located all over the body with clusters in the neck, armpits and groin and are populated mainly by lymphocytes. The right lymphatic duct and the thoracic duct drain lymph fluids into two veins that come together to form the inferior vena cava, which passes into the heart. The cisterna chyli is a widened portion of the thoracic duct, where fluids from several lymph-collecting vessels are received. The spleen also removes and destroys worn-out red blood cells, however it is involved in the fight against infections.

### **Bone marrow**

Haematopoiesis usually takes place in bone marrow within the long bones.

Bone marrow is organized in haematopoietic and non-haematopoietic compartment, interspersed in meshwork of mesenchymal or stromal cells in the spine and the cavity of long bones. In fact, the mesenchymal cells and osteoblasts secrete supporting factors necessary for the survival of mesenchymal stem cells which undergo a process called haematopoiesis. During haematopoiesis, bone marrow-derived mesenchymal stem cells differentiate into either mature cells of the immune system or into precursors

of cells that migrate out of the bone marrow to continue their maturation elsewhere. The bone marrow produces B and T cells, granulocytes and monocytes, in addition to red blood cells and platelet.

B-lymphocytes (B-cells) are produced in the bone marrow and require bone marrow stromal cells and their cytokines for maturation. During its development, each B-lymphocyte becomes genetically programmed through a series of gene-splicing reactions to produce an antibody capable of binding a specific epitope of an antigen. Antibodies on the surface of B-lymphocytes (surface immunoglobulin, sIg) function as B-cell receptors. In fact B-lymphocytes are able to recognize soluble antigens as well as microbes such as viruses bound to the B-cell receptor. In addition, B-lymphocytes appear to survey antigen-presenting dendritic cells and acquire antigens from their surface. The first signal for the activation of a naive B-lymphocyte occurs when B-cell receptors on the surface of the B-lymphocyte bind epitopes of antigens having a corresponding shape. A second signal is also needed for the activation of the naive B-lymphocyte. This is provided when a component of the complement system called C3b binds to the microbial surface. C3b is subsequently degraded to C3d which, in turn, binds to a complement receptor called CR2 on the surface of the B-lymphocyte. Subsequently, the antigen is phagocytosed and degraded with lysosomes. During this process, protein antigens are degraded into a series of peptide epitopes.

These peptides eventually bind to MHC-II molecules that are carried to the surface of the B- cell.

In immune response B cells express immunoglobulin (Ig) and class II MHC molecules on their cell surface. Furthermore, B lymphocytes are capable of producing antibody with the same specificity as that expressed by their immunoglobulin receptor; in addition they function as an antigen presenting cell to a helper T cell. Activation of the T cell results in the production of cytokines that enable the specific B cell to become activated to produce soluble antibodies. Binding of an antigen to the immunoglobulin receptor delivers one signal to the B cell, but that is insufficient. Second signals delivered by costimulatory molecules are required; the most important of these is

CD40L on the T cell that binds to CD40 on the B cell to initiate delivery of a second signal.

## Lymph nodes

Lymph nodes are small encapsulated organs distributed along the pathway of lymphatic vessels of the body, with large concentrations occurring in the areas of convergence of lymph vessels. (Fig 1) The diameter may vary from about 1 mm to 1 cm. They serve as filters through which lymphocytes tread on its way to the blood. They consist of an outer cortical and an inner medullary part.

The parenchymal is characterized by the presence of lymphoid follicles that results be constituted by germinal center (light zone) and mantle zone (dark zone).

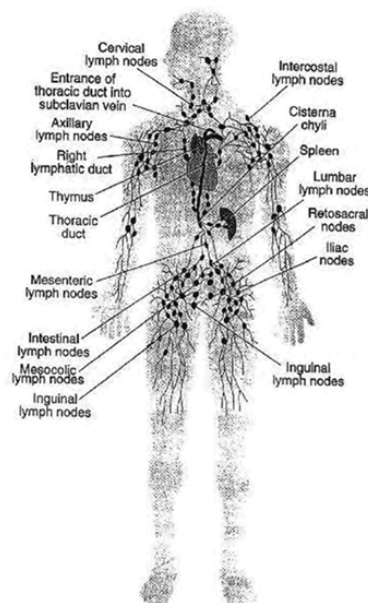


Fig.1 Anatomic location of lymph nodes in human body

## **The thymus**

The thymus is a lymphoid organ, within thoracic cavity, that is entirely formed and functional at birth. It is the organ where immature lymphocytes undergo maturation. It persists until about puberty, when lymphocyte processing and proliferation are dramatically reduced and it is largely replaced by adipose tissue. The lymphocytes released by the thymus are carried to lymph nodes, spleen, and other lymphatic tissue where they form colonies. In particular, T- lymphocytes survive for long periods and recirculate through lymphatic tissues. Occasionally the thymus persists after puberty and the production of abnormal T-cells may contribute to development of some autoimmune disorders.

## **The spleen**

The spleen performs the function of filter and it reacts immunologically to blood-borne antigens. Microscopically, it is composed of red and white pulp. White pulp in the spleen contains lymphocytes and is equivalent to other lymphoid tissue, while red pulp contains large numbers of red blood cells, which are filtered and degraded here. In addition to large numbers of lymphocytes, the spleen contains specialized vascular spaces, a meshwork of reticular cells and fibers, and a rich supply of macrophages which monitor the blood. Connective tissue forms a capsule and trabeculae which contain myofibroblasts, which are contractile. The spleen functions in both the immune and the hematopoietic systems. Immune functions include removal of antigens from the blood, proliferation of lymphocytes and production of antibodies while hematopoietic functions consist of formation of blood cells during fetal life, removal and destruction of aged, damaged and abnormal red cells and platelets, retrieval of iron from hemoglobin degradation and storage of red blood cells.

## **Diffuse Lymphatic Tissue and Lymphatic nodules**

The lymphatic tissue may be detected in connective tissue beneath the epithelial mucosa in the alimentary canal, the respiratory passages, and also genitourinary tract. It is responsible for the intercept foreign antigens and subsequently go to lymph nodes to undergo differentiation and proliferation. Lymphocytes constituted in microaggregates and forme the lymphatic nodules.

The lymphatic nodules are localized random in the body even if are more concentrated in the GI tract in the ileum, appendix, caecum, and tonsils. They are called the Gut Associated Lymphatic Tissue (GALT) and MALT (Mucosa Associated Lymphatic Tissue).

## T AND B-CELL DEVELOPMENT

T cells arise from progenitor lymphoid cells in the bone marrow and mature during migration through the thymus to become either CD4+ or CD8+ single positive cells via double negative (CD4- and CD8-) and double positive (CD4+ and CD8+) thymocytes. TCR V(D)J gene rearrangements occurs in the thymus and structural differences enable the distinction of  $\alpha\beta$  (vast majority of all T cells) and  $\gamma\delta$ T cells. <sup>(6)</sup>

After activation of the TCR, T cells proliferate and differentiate into memory T cells (CD4+) or effector T cells (CD8+). T cells with non-functional TCRs, low antigen affinity or self reactive TCRs die by apoptosis. <sup>(7)</sup>

B cells arise and differentiate in the bone marrow. During this differentiation, IgH and IgL V(D)J gene rearrangements result in the generation and surface expression of membrane bound antibodies capable of binding antigen.

Mature naive B cells flow in the peripheral blood, populate primary lymphoid follicles and are activated by antigens. Antigen exposed B cells migrate into the center of primary follicles, proliferate and form together with the follicular dendritic cell (FDC) meshwork a germinal center (GC).

Somatic hypermutation in the IgH and IgL region genes and class switch recombination are introduced during the GC reaction to improve antigen affinity. <sup>(8)</sup>



## **FUNCTION OF LYMPHOCYTES IN IMMUNE SYSTEM**

The human immune system can be defined as an interactive network of cells and molecules with specialized roles in defending the body against pathogens. There are two different types of immune response: the innate or natural immune response and the adaptive or acquired immune response. The innate immune response occurs unspecific as a first line defence against pathogens.

Various cells are mainly involved in the innate immune response, even if they have a limited number of receptors to recognize pathogens. <sup>(9)</sup> The cellular elements involved in the innate immune response are phagocytic cells (neutrophils and macrophages), inflammatory mediators releasing cells (basophils, mast cells, and eosinophils), and natural killer (NK) cells.

The adaptive immune response is able to fight the wide range of pathogens. In fact cells that modulate this type of response, such as lymphocytes B and T, recognize a great variety of different viral and bacterial antigens. <sup>(7)</sup>

The main characteristic of B cells is the expression of specific antigen binding immunoglobulin (Ig) proteins, also known as antibodies. Membrane bound antibodies on the B cell surface are part of the B cell receptor (BCR) complex.

Antibodies consist of two identical immunoglobulin heavy chains (IgH) and two identical immunoglobulin light chains (IgL) that are linked by disulfide bonds. The variable antigen binding region (V region) at the N-terminal of each chain varies extremely between different antibody molecules, whereas the constant region (C region) at the C-terminal consists of only a few subtypes and is responsible for activating different effector mechanisms. <sup>(10,11)</sup>

Secreted antibodies are able to bind specifically pathogens and recruit other cells (e.g. phagocytes) to eliminate the pathogen and to bind at the V region of the BCR complex that leads to B-cell activation and to the production of antibodies with higher affinity. <sup>(7)</sup>

The main function of T cells is the identification and elimination of other immune cells infected by pathogens. Instead T cells recognize antigens through membrane proteins present in their receptors. T cell receptors (TCRs) consist of  $\alpha/\beta$  (vast majority) or  $\gamma/\delta$  heterodimer that also contain V and C regions. In contrast to the BCR, TCRs are not able to bind antigens directly. They recognize short peptide fragments of protein antigens processed by the ubiquitin system, which are present by the major histocompatibility complex (MHC) molecules on the cell surface. <sup>(12,13)</sup>

## GERMINAL CENTRE MICROENVIRONMENT

The exposure to a microbe or an antigen induces the activation of naïve B lymphocytes, which may be dependent or not by T cell.

In the T cell dependent maturation process the CD4<sup>+</sup> helper T lymphocytes stimulates B cells leading to a humoral immune response with antibodies of high affinity against protein antigens. In contrast, antibodies response to non-protein antigens does not need participation of a helper T cell generating antibodies with a lower affinity for the antigen. T cell dependent antigen responses can lead to two different B cell maturation processes. In fact, naïve B cells can be induced to proliferate and differentiate in either follicular or extra-follicular antibody-secreting cells. T cell dependent extrafollicular responses provide early antibody, which can be critical for halting the spread of viral infection. In this condition, memory B cells are not produced. By contrast with the establishment of a primary follicle and, successively, a GC, naïve B cells differentiate and show increasing affinity maturation of the BCR. <sup>(14)</sup>

GC is formed from a small number of IgM<sup>+</sup> IgD<sup>+</sup> B cells in the follicles of peripheral lymphoid tissues (spleen, lymph node, Peyer's patches and tonsils). Under experimental conditions, GCs initially form approximately 6 days after a primary immunization, when foci of rapidly proliferating B cells begin to appear within the B cell follicles of lymph nodes and spleen.

Naïve B cells that have taken up antigen through their BCRs, internalize it and after digestion expose it through the MHC class II (major histocompatibility complex class II) molecules. Thus, naïve B cells migrate into lymphoid tissue through high endothelial venules (HEV) and at first enter the T cell zones. Here, antigen-binding B cells are selectively trapped to maximize the chance of encountering a CD4<sup>+</sup> helper T cell that can activate them. This interaction is managed by MHC class II molecules on the surface of B cells associated with the antigen that is recognized by T cell receptor

(TCR) on T cells surface.

Helper T cell that recognizes MHC: peptide complex on B cell also binds to B7 molecule with its CD28 ligand and gets stimulated to proliferate. BCR signalling is also enhanced by the presence of another important surface molecule CD40, which is a member of the tumor-necrosis factor (TNF)- receptor family that is expressed by all B cells. CD40 receptor binds to its ligand CD154 (or CD40L) expressed by CD4+ helper T cells. This engagement results in the nuclear translocation of NF- $\kappa$ B that stimulates proliferation. Mice with no functional CD40 or CD40L do not develop GCs, and mechanistic disturbance of this interaction leads to a dissolution of formed GCs.<sup>(15)</sup> Once activated, B cells move through the T cell zone into the B cell zone where they start to proliferate forming a primary follicle of clonal expansion. For several days B cells massively continue to proliferate forming the GC.

In addition to interaction with B cells, T cells are stimulated to produce cytokines such as IL-4 that together to CD40L promote the clonal expansion that precedes antibody production. Thus, the combination of BCR and CD40 ligation, along with IL-4 and together with others signals derived from T cell contact, lead to B cell proliferation.

B cells that form GC are called centroblast. In this GC phase, centroblasts start to express BCL6, an important GC factor, and continue to proliferate, forming the characteristic phenotype of the GC with two visible zones: the dark and the light zones.<sup>(16)</sup>

In the dark zone B cells are blasts which do not express CD40 molecule. In fact, dark zone is largely devoid of T cells that express CD154 (CD40L molecule), and then is not necessary for B cells to expressed CD40 surface molecule, and the interaction between CD40 and CD154 does not occur.<sup>(17)</sup>

NF- $\kappa$ B may be expressed or not a very low levels, in fact NF- $\kappa$ B is a repressor of BCL6 that is one important regulator of the early phase of GC.

Dark zone is so called because the proliferating B cells are densely packed, while the light zone is more supplied with follicular dendritic cells (FDC), follicular helper T cell (TFH) and macrophages but less densely packed with B cells.

The high proliferation rate of centroblasts is required for the generation of large number of modified antibodies, and to allowed the selection of few B cells that display antibodies with improved antigen-binding specificity. Importantly, in the dark zone of centroblasts somatic hypermutation (SHM) takes place and in this process the V region of Ig genes may be altered leading to a high number of “new” B cells.

Gene expression profile analysis has shown that the differentiation of an antigen-activated B cell into a centroblast is accompanied by a dramatic up-regulation of genes associated with cell proliferation and the down-regulation of genes encoding negative regulators of clonal expansion, such as p21. <sup>(18)</sup>

DNA damages frequently occur, and in this condition in high proliferating B cells response are specifically suppressed. <sup>(19)</sup>

Compared to centroblast, centrocytes are very heterogeneous and must undergo selection, further DNA modification and initiation of differentiation into PC or memory B cell. In the light zone selection of B cells expressing mutated BCR is essential to generate antibodies with high-potential affinity.

Current evidence favours a two-stage process for centrocytes selection. First, the cells engage antigen through their BCR. In this phase the antigen is exposed by FDC and the selection is controlled by direct signals derived from BCR cross-linking more strong in proportion to the affinity of the BCR. <sup>(20)</sup>

This selection phase is thus based on affinity-like parameter that change with mutations.

The second phase seems to involve the binding between CD40 expressed on centrocytes and CD40L expressed by TFH.

Thus, in the light zone CD40 molecule is re-expressed on centrocytes leading to signals of “delivery of survival”.

B cells with a too low affinity for the antigen die because are not rescue by CD40 signal. These signals provide the cell fate of mature B cells with the activation of the NF-kB factor that inhibits BCL6 and GC important genes and up-regulates differentiation pathways.

Class switch recombination (CSR) is also induced leading to DNA modification with which B cells change Ig class expression from IgM and IgD to other classes that have distinct effectors function (IgG, IgA and IgE). Therefore, various cytokines, expressing by TFH, play an important role in regulating the pattern of heavy-chain isotype switch. For example, IL-4 induces switch to IgE. Dark zone and light zone are two GC compartments containing B cells that are usually seen as two different support the idea that centroblasts and centrocytes are the same B cells which differed for their status.

(21)

Gene expression profile analysis reveals that, rather than differentiation pathways, centroblasts and centrocytes differ for proliferation pathways more active in the dark zone, and for signalling pathways more active in the light zone where B cells encounter FDC and THF.

## LYMPHOMAS

Lymphomas are clonal tumors of mature and immature B, T or natural killer (NK) cells at various stages of differentiation and are classified according to the WHO classification of lymphoid neoplasms. <sup>(22)</sup>

Lymphoid neoplasms represent approximately 4% of new cancers per year worldwide, are more common in the Western World and contrary to most other common neoplasias, have been increasing in incidence worldwide. <sup>(23)</sup>

In many aspects, B- and T-cell lymphomas appear to recapitulate stages of normal differentiation, so they can be classified according to the corresponding to their physiological equivalents.

Based on morphological features, two major groups of malignant lymphomas have been distinguished: classical Hodgkin lymphoma (cHL) and non-Hodgkin lymphoma (NHL) which comprise over 90% of these. <sup>(22)</sup>

Non-Hodgkin lymphoma (NHL) originating from mature B cells represents a very morphological and molecular heterogeneous malignancies with different clinical behaviour and biology and include several lymphoma subtypes.

NHL have incidence rates over 10/100.000 in the USA, Australia and Europe. Lower rates of less than 5/100.000 are reported in Central America and parts of Africa. <sup>(24)</sup>

Interestingly, the vast majority of NHLs originates from B cells. <sup>(22)</sup>

Biologically, NHLs may present in an indolent form (or low-grade lymphoma) characterized by slow growth and in aggressive forms (or high-grade lymphoma) with rapidly course which requires prompt treatment. <sup>(25)</sup>

All NHLs are derived from B-lineage cells that have completed both immunoglobulin (Ig) heavy-chain and light-chain (IgH and IgL) recombination and can express functional Ig protein. Many evidences highlight that the promoting of tumorigenesis of B and T lymphocytes derives from genomic alterations such as translocations,

chromosomal gains and losses as well as mutations in context-specific oncogenes or tumour suppressors. <sup>(26-30)</sup>

However, within the last decade it has become increasingly evident that changes in the epigenetic landscape, i.e. changes in chromatin and methylation patterns, are also frequently observed in B cell malignancies. <sup>(31-32)</sup>

In the several B-NHL neoplastic cells recapitulate the normal stages of differentiation so they can be classified according to their normal counterpart. However, some neoplasms do not have as yet a well-defined normal counterpart and show morphological, immunophenotypical and molecular heterogeneity.

In fact, most of subtypes of NHLs may be distinguished using traditional clinical and pathological methods <sup>(33)</sup>, however others require Gene Expression Profiling to be diagnosed. <sup>(34)</sup>

Each lymphoma subtype bears a phenotypic resemblance to B cells at a particular stage of differentiation, as judged by the presence or absence of immunoglobulin (Ig) variable (V) region mutations and by gene expression profiling. The normal B cell counterpart is considered as the “cell of origin” of a lymphoma.

The signaling pathways that normal B cells deploy to sense antigens are frequently derailed in B cell malignancies, leading to constitutive activation of prosurvival pathways.

The mature B cell malignancies represent a medical challenge that is only partly met by conventional therapy, justifying concerted investigation either into their molecular circuitry and pathogenesis and into stromal and immunological tumor-microenvironment. Recent insights into lymphoma pathogenesis are leading to the rational development of targeted therapies that may achieve appropriate therapies.

For this study Splenic Marginal Zone Lymphoma and Diffuse Large B Cell Lymphoma (DLBCL) are of interest and thus subsequently described in more detail.



## Splenic Marginal Zone Lymphoma

Splenic Marginal Zone Lymphoma (SMZL) is a rare low-grade B cell disorder representing 2% of all non-Hodgkin lymphomas (NHL).<sup>(35)</sup>

For the first time Schmid used this term to describe cases of lymphoma arising in the splenic marginal zone.

In some patients, SMZL is occasionally diagnosed after the detection of peripheral lymphocytosis, however in advanced-stage it is characterized by symptomatic splenomegaly with abdominal pain and cytopenia. Around 20% of patients present also autoimmune disease.<sup>(36)</sup>

Although the majority of patients show an indolent course with a median survival of approximately 9 years<sup>(37)</sup>, the prognosis of SMZL is heterogeneous. Indeed, 30% of patients present an unfavourable outcome<sup>(38)</sup>, including 5% to 10% of patients undergoing transformation to diffuse large B-cell lymphoma.<sup>(39)</sup>

SMZL is characterized by the constant involvement of the red and white pulp of spleen, peripheral blood and very often also bone marrow. (Fig 2)

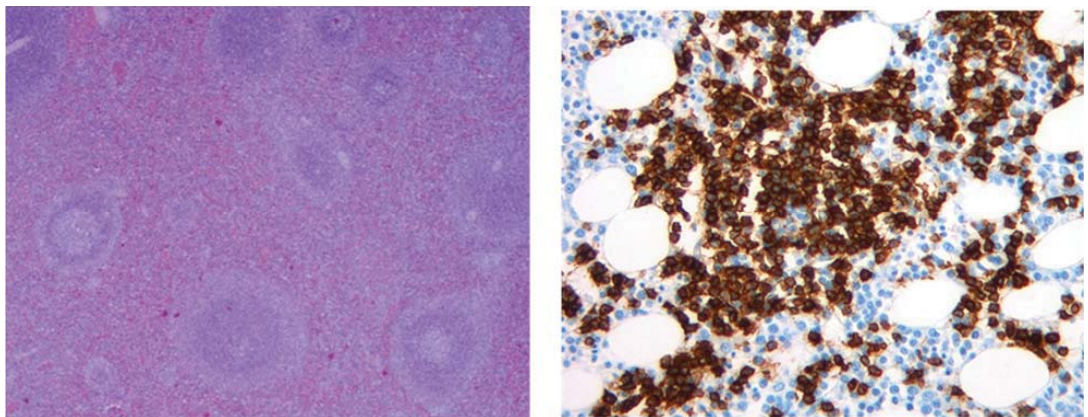


Fig 2 The panel on the left shows the histological features of SMZL; the panel on the right shows the BM involvement of SMZL by CD20+ IHC staining.

SMZL occurs mostly in middle-aged and elderly patients and there seems to be an

equal sex incidence.<sup>(35,40)</sup> SMZL is defined an indolent lymphoma for a rather indolent course, however in a third of cases it presents an aggressive course.

In most patients splenectomy alone or followed by chemotherapy is the elective treatment. This entity in fact has a favourable prognosis with a 5-year survival of 65-75%<sup>(41,42)</sup>

In the WHO 2001 classification of tumours of haematopoietic and lymphoid tissues, SMZL has been recognized as an entity with histological and immunophenotypical features different from the other low-grade B-cell lymphomas.<sup>(43)</sup>

The spleen usually has a gross weight of more than 400 g and the cut surface shows a typical micronodular aspect.

SMZL develops in the white pulp with a biphasic picture. Medium- size monocytoid B cells are organized into a pale ring around the follicle, whereas small centrocyte-like cells efface the mantle zone and colonize the germinal centers. In SMZL a plasmacytic differentiation is present in most cases. The neoplastic proliferation involves also the red pulp with patch or diffuse infiltration pattern. It is also detected the presence of large cells presenting immunoblastic morphology which increase in that cases of disease progression. In bone marrow biopsy, an intrasinusoidal characteristic pattern is observed together to interstitial one.

SMZL do not present a specific immunophenotype profile. It is characterized by IgM and IgD co-expression and lacks expression of CD5, CD23 and CD10.<sup>(35)</sup>

Splenic lymphoma with villous lymphocytes (SLVL) was defined by Catovsky based on the presence of atypical B-lymphocytes with villous cell membranes in the peripheral blood of a category of patients with splenomegaly.<sup>(44)</sup>

It is now generally accepted that most cases of SLVL represent a leukemic counterpart of SMZL.<sup>(45)</sup>

Lymph nodes (LN) of patients diagnosed with SMZL show a mantle zone pattern, rather than a marginal zone like histology. This pattern clearly differs from other MZL like extranodal marginal zone lymphoma (MALT lymphoma) or monocytoid B-cell lymphoma where the neoplastic cells are present outside the follicles surrounding the

preexisting mantle zone or are present in a parafollicular or sinusoidal pattern. Moreover, other lymphoid malignancies such as follicular and mantle cell lymphomas infrequently infiltrate the marginal zone resembling SMZL.<sup>(46)</sup> In bone marrow SMZL presents a typical intrasinusoidal pattern of infiltration which is useful for differential diagnosis with other lymphoproliferative disorders. (Fig 3)

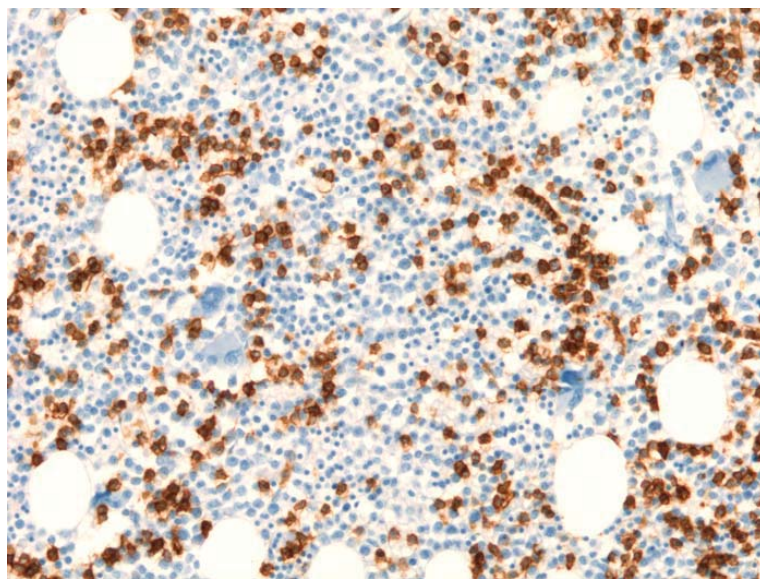


Fig 3 CD20 positive lymphomatous cells lodged in the distended lumina of the bone marrow sinusoids in SMZL

SMZL lacks expression of CD5 and CD10 like the other types of MZL and normal splenic marginal zone B cells.<sup>(47,48)</sup>

Several groups have investigated to identify the normal counterpart of SMZL by analyzing IgH chain gene rearrangements. Some authors have demonstrated that SMZL have a (post)-germinal center origin.<sup>(49)</sup>

Other studies suggest that SMZL may originate from either pre-germinal center B-cells with unmutated VH genes, (post)-germinal center memory cell with mutated VH genes or germinal center B cells with ongoing somatic mutations. Alternatively, it has been

proposed that somatic mutations of the Ig genes are not confined to the germinal center environment.

## **Diffuse Large B Cell Lymphoma**

Diffuse Large B cell Lymphoma (DLBCL) is a high-grade lymphoproliferative disorder characterized by the proliferation of lymphoid cells of large size, which harbor a B cell phenotype.

DLBCL is the most common Non-Hodgkin Lymphoma (NHL), accounting for 35%-40% of all cases. <sup>(50)</sup>

DLBCL may originate in a nodal (lymphoid) or extranodal site, including unusual sites, such as testis, bone, and lung, and present an aggressive behaviour, even if it is highly responsive to chemotherapy.

DLBCL can also develop in the setting of immunodeficiency, primary or acquired one. <sup>(51)</sup>

DLBCL usually arises de novo but can represent transformation of an indolent lymphoma, such as follicular lymphoma (FL) <sup>(52)</sup>, chronic lymphocytic Leukaemia/Small Lymphocytic Lymphoma (CLL/SLL) in the so-called Richter's transformation <sup>(53)</sup>, and marginal zone lymphoma (MZL) or nodular lymphocytic predominant Hodgkin lymphoma (NLPHL). <sup>(54)</sup>

The therapeutic treatment consists the combination of Anthracycline-based combination polychemotherapy (CHOP) with anti-CD20 monoclonal antibody rituximab.

Nevertheless, 50% of the DLBCL patients cannot be cured yet because of primary refractory disease or relapses after first-line therapy. <sup>(55)</sup>

Before the introduction of rituximab, the International Prognostic Index (IPI) has been useful to predict the response to treatment and prognosis of patients with DLBCL. This stratification into four risk groups with 5-year over- all survival (OS) ranging from 73% in the most favorable group to 26% is based on risk factors (age older than 60

years, poor performance status, elevated LDH, advanced Ann Arbor stage, and presence of more than one extranodal site) in patients with aggressive NHL. <sup>(56)</sup>

However, the combination of rituximab to standard CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine, prednisolone) has led to a marked improvement in survival and has restricted the outcome differences between the risk groups. <sup>(57)</sup>

Patients with DLBCL present a variability in the outcome. This variability reflects the remarkable heterogeneity of this disease which can be appreciated in its variable clinic, morphology, immunophenotype, and genetic.

Gene expression profiling (GEP) has allowed the different DLBCL subtypes with different prognostic significance. <sup>(58)</sup>

Two subtypes of DLBCL are identified based on the cell of origin with different clinical, biologic and genetic characteristics <sup>(59)</sup>: germinal center B cell-like (GCB) DLBCL, expressing genes that are hallmarks of normal germinal center B cells and activated B cell-like (ABC) which lacks the expression of germinal center B cell-restricted genes and possibly arising from post-germinal center B cells that are arrested during plasmacytic differentiation.

GCB-DLBCLs can origin from highly proliferating B cell located within the GCs, in fact this DLBCL molecular

subgroup harbors the typical expression pattern of GC B cells such as CD10 and BCL6 gene expression. ABC-DLBCLs may arise from post-GC B cells that are arrested during plasmacytic differentiation as suggest by the expression of NF-kB target genes caused by NF-kB constitutive activation.

## MICROENVIRONMENT IN B-NHLS

The germinal centers (GCs) of lymphoid follicles contain two different micro environmental zones: the “dark” zone and the “light” zone. The “dark” zone at the base of the follicular center contains tightly packed centroblasts, whereas the “light” zone contains small mature cells called centrocytes. The follicular dendritic cell (FDC) network extends throughout the GC, however it may be much detected in the dark than in the light zone. Some CD4 T lymphocytes are found in the light zones. Within the GC the fibroblastic reticulum cells (FRCs) and Tingible Body Macrophages (TBMs) are present. FRCs make a sharp demarcation around the follicle center while TBMs are located in the basal light zone. <sup>(60)</sup>

Neoplastic clone lymphoid cells habitat and proliferate in association with an environment that maintain the characteristics of normal GC cellular microenvironment. Many studies have demonstrated the role of microenvironment in the pathogenesis and prognosis in follicular lymphoma. Gene expression profiling analyses have been confirmed by immunohistochemical and flow cytometry data, showing the presence of the main components of the anti-tumor immune response, such as specific CD4+ T-helper lymphocytes <sup>(61,62)</sup>, CD8+ T lymphocytes <sup>(63)</sup> and CD68+ macrophages. <sup>(64)</sup>

Moreover, altered immune profiles in the tumor microenvironment of FL determined a reprogramming of tumor-associated macrophages. The polarized M1 and M2 macrophages perform either antitumor cytotoxic activity or protumoral activity, promoting tumor growth and progression. <sup>(65)</sup>

In FL, the neoplastic proliferation forms follicular structures together with helper T cells and FDCs. <sup>(66,67)</sup>

Neoplastic follicles in FL contain, in addition to FDC (vimentin+, CD21+, CD23+) other non-neoplastic cells normally observed in GC including macrophages (CD68+) and GC T cells (CD31, CD41, CD57+, PD1+).



In most cases the differential diagnosis of the GC-derived lymphoid malignancies from their reactive counterparts (e.g. reactive hyperplasia) is appreciable and evaluated through their microenvironment features.

In approximately 85% of grade I and grade II FL, GC cells express BCL2 protein. The remaining 15% of grade I and grade II FL and about the 50% of grade III FL are BCL2 negative.

Furthermore, GEP analysis confirmed that the prognostic signatures in follicular lymphoma are not expressed by immunological cells of microenvironment (T cells, macrophages, or dendritic cells) but not by the neoplastic clone.

The influence of the cellular microenvironment on the outcome of FL probably reflects the active and considerable participation of immune cells either in the biology and in the pathogenesis of this subtype of lymphoma.

In follicular lymphoma, the histologic features resemble the structure of normal germinal centers with the distinctive association with T-Helper cells, FDCs, and TAM in the neoplastic follicles.

The microenvironmental influences play also a crucial role in disease progression, as well as in resistance to treatment in Chronic Lymphocytic Leukemia (LCC).

Stamatopoulos et al further have demonstrated that a favorable microenvironment may favour the lymphomagenesis and disease progression. <sup>(68)</sup>

On the contrary, unfavourable microenvironmental conditions may impaired the neoplastic proliferation and the molecular mechanisms interacting with antiapoptotic signals.

CXCR4 is expressed by the most circulating CLL neoplastic clone cells at high levels, independently from molecular markers, clinical stages or patterns of bone marrow infiltration.

BM stromal cells constitutively secrete CXCL12, the ligand of CXCR4. Consequently, the CXCL12/CXCR4 axis plays a crucial role in the recruitment of neoplastic cells to growth-favorable environments.

LLC neoplastic clone can also secrete chemokines inducing stromal remodeling by

recruiting accessory cells and creating a prone microenvironment to neoplastic growth. Moreover, increased angiogenesis has been consistently associated with more advanced disease phases and unfavorable outcome.

It is known that the neoplastic cells promote the neoangiogenesis, being able to secrete pro-angiogenic factors, including fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and angiopoietin (Ang). These microvessels are characterized by increased permeability, and thus contribute to disease progression.

GEP studies have contributed to the identification of two biological subtypes of DLBCL.

The microenvironment has an important role in pathogenesis of this lymphoproliferative disorder. In fact the lymphomagenesis in fact seems to be linked to BRC proliferation and host response. This latter is determined by activated T/NK cells, macrophages, (S100+) dendritic cells and stromal cells, that have a protumoral or antitumoral action. <sup>(69)</sup>

In GEP analysis the two subtypes ABC/GCB of DLBCL show different “lymph node signature” that include information regarding macrophage and extracellular e seem to have a prognostic value, suggesting a cytotoxic response. <sup>(70)</sup>



## **AIMS OF THE THESIS**

My project research is aimed on dissecting the tumour-associated immunological and stromal microenvironment in B-cell malignancies, in particular in SMZL and in DLBCL.

The general aims of this PhD project were to dissecting the tumour-associated immunological and stromal microenvironment in B-cell malignancies to identify new tools that could enable more accurate diagnosis in early stage of disease, potential prognostic markers and also therapeutic targets.

In this setting I have investigated the reciprocal influence of immune cells and stromal elements on the development and progression of Splenic Marginal Zone Lymphoma (SMZL), where the pathogenesis is related to a deregulated immunologic stimulation, by focusing on the microenvironment of the bone marrow (BM), which represents an elective disease localization endorsing diagnostic and prognostic relevance.

I have also investigated the stromal and immunological compartments of microenvironment to identify new prognostic and therapeutic biomarkers in DLBCL. Therefore, I have examined the tumor microenvironment, recognizing it as a key factor in multiple stages of disease progression and a pathological active niche that shapes tumor evolution.

## **MATERIALS AND METHODS**

### **Pathological samples**

For this present study, 66 cases of SMZL in asymptomatic phase were selected from the archives of the department of Pathology of the University of Palermo, Pavia and Bologna, all diagnosed according to WHO criteria.

### **Immunohistochemical analysis**

Immunohistochemistry was performed using a polymer detection method. Briefly, tissue samples were fixed in 10% buffered formalin and paraffin embedded. Four-micrometers-thick tissue sections were deparaffinized and rehydrated.

The antigen unmasking technique was performed using Novocastra Epitope Retrieval Solutions pH6, pH8 and pH9 in PT Link Dako 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% H<sub>2</sub>O<sub>2</sub> and Fc blocking by a specific protein block (Novocastra UK) the samples were incubated 1h with the primary antibodies Mouse anti-human monoclonal CD163 (clone 10D6) 1/100 pH6 Leica Biosystems Newcastle Ltd; CD33 (clone PWS44) 1/100 pH 9 Leica Biosystems Newcastle Ltd; CD23 (clone 1B12) 1/50 pH 6 Leica Biosystems Newcastle Ltd; CD21(clone 2G9) 1/20 pH 6 Leica Biosystems Newcastle Ltd; CD20 (clone L26) 1/100 pH 9 Leica Biosystems Newcastle Ltd ; CD8 (clone 1A5) 1/20 pH 6 Leica Biosystems Newcastle Ltd; CD5 (clone 4C7) 1/100 pH 9 Leica Biosystems Newcastle Ltd; IgD (clone DRN1C) 1/1000 pH 9 Leica Biosystems Newcastle Ltd; IgM (clone 8H6) 1/800 pH 9 Leica Biosystems Newcastle Ltd; Bcl-6 (clone LN22) 1/100 pH 9 Leica Biosystems Newcastle Ltd; FoxP3 ((clone 236A/E7) 1/100 pH 9 Abcam; PD1 (clone NAT105) 1/50 pH 8 Abcam; PD-L1 (clone 22C3) 1/50 pH 9 Dako; Collagen

Type I (clone 3G3) 1/100 pH 9 Acris; Collagen Type IV (clone 24.12.8 PMH-12) 1/80 pH 9 Millipore; CD134 OX40 (clone ACT-35) 1/50 pH 6 eBioscience ; Osteonectin/SPARC (clone ON1-1) 1/500 pH 8 Takara; Rabbit anti-human monoclonal Osteopontin (clone EPR3688) 1/100 pH 9 Abcam; Rabbit anti-human polyclonal Arginase 1, 1/200 pH 9 Genetex; at room temperature.

Staining was revealed by polymer detection kit (Novocastra) using either AEC (3-amino-9-ethylcarbazole) Dako, or DAB (3,3'-Diaminobenzidine, Novocastra) substrate-chromogen. The slides were counterstained with Harris hematoxylin (Novocastra).

All the sections were analyzed under a Zeiss AXIO Scope.A1 optical microscope (Zeiss Oberkochen Germany) and microphotographs were collected using a Zeiss Axiocam 503 Color.

## **Animals**

All mice are 8 to 10 weeks of age and were purchased from Charles River Laboratories. Both control mice BALB/c AnNCrl, in pathogen-free conditions and mice Fas lpr/lprOPN <sup>-/-</sup> were maintained in animal facility of the Fondazione IRCCS Istituto Nazionale dei Tumori (Milan, Italy).

The institutional ethics committee for animal use authorized all animal experiments.

## **Evaluation of Autoimmunity and Lymphoproliferation in Fas lpr Mice**

Swelling of SLO was evaluated by caliper measurement, whereas histopathologic and immunophenotypic analyses were performed according to the criteria for lymphoid neoplasm classification. <sup>(71)</sup>

### **Statistical analysis**

Statistical analysis was performed using the SPSS for Analytics. Values were considered statistically significant when  $p < 0.05$ . All of the analyses were performed using Prism software Version 5.0d (GraphPad).

## RESULTS AND DISCUSSION

All cases of SMZL enrolled as described in materials and methods, present the following relevant clinical characteristics as summarized in Table 1.

Characteristic	No. of patients	(%)
<b>Age at diagnosis (range)</b>	<b>66 (35-84)</b>	
<b>Sex</b>		
Male	26	(48.1)
Female	28	(51.9)
<b>Intergruppo Italiano Linfomi (IIL) Score</b>		
Low risk	21	(40.4)
Intermediate risk	15	(28.8)
High Risk	16	(30.8)
<b>Splenectomy</b>		
Yes	10	(21.3)
No	37	(78.7)
<b>Disease Progression</b>		
Yes	35	(64.8)
No	19	(35.2)
<b>Treatment at diagnosis</b>		
Watchful waiting	24	(46.2)
Other treatment (Splenectomy and/or Chemotherapy)	28	(51.9)
<b>Type of treatment at diagnosis</b>		
Chop like regimen (without Rituximab)	8	(30.8)
Chop like regimen (with Rituximab)	7	(26.9)
Alkylating agents	2	(7.7)
Purine analogues	6	(23.1)
Splenectomy	3	(11.5)
<b>Laboratory features</b>	<b>Mean</b>	<b>Range</b>
Hemoglobin (g/dl)	11.85	(7-17)
White Blood Cell (WBC) count (x 10 <sup>9</sup> /L)	15.4	(1.8-91)
Lymphocytes (x 10 <sup>9</sup> /L) count	11.3	(0.3-77.3)
Platelets (PLT) count (x 10 <sup>9</sup> /L)	151.8	(51.2-380)
Lactate dehydrogenase (LDH) (U/L)	472	(186-1482)
Albumin (g/dl)	3.8	(2.9-5.1)
<b>Pathological features</b>		
Percentage of lymphoid infiltration (%)	35	(5-80)
Residual BM hemopoietic cellularity (%)	59	(30-90)
	<b>No. of patients</b>	<b>(%)</b>
<b>Pattern of bone marrow infiltration</b>		
Nodular	3	(8.5)
Interstitial	5	(14.3)
Intra-sinusoidal	3	(8.5)
Mixed (Nodular ± Interstitial ± Intra-sinusoidal)	24	(68.7)
<b>CD10+ ARC meshwork *</b>		
Low	23	(65.7)
High	12	(34.3)
<b>CD31+ microvessel density **</b>		
Low	18	(51.4)
High	17	(48.6)
<b>CD40+ stromal cell meshwork *</b>		
Low	33	(61.1)
High	21	(38.9)

\* Semi-quantitative analysis: low are considered cases with score 0-1 and high cases with 2-3 score; \*\* Low are considered cases with amount of CD31+ vessels below the median value; High are cases with amount of CD31+ vessels above the median value.

The peripheral smear and the histological bone marrow features are reviewed and also the immunohistochemical profile was evaluated to confirm the diagnosis of lymphoma marginal.

For SMZL the Intergruppo Italiano Linfomi (IIL) has identified a clinical prognostic score which is able to

stratify patients into three different risk categories based on three parameters assessed on diagnosis - anemia, elevated LDH levels and hypoalbuminemia.

Once applied to this score, all patients were stratified in high, intermediate and low risk.

Subsequently we wanted to test whether features inherent with the peripheral blood analysis were associated with immunophenotypic clonal expression and if any correlations exist between these variables.

To this end we quantified the density of the CD5 on lymphomatous cells and the expression of the heavy chains (IgM and IgD). Furthermore, on multivariate analysis, we detected a correlation between the expression of these markers and IIL score parameters.

Among the variables tested, we showed a correlation between low Hemoglobin concentration and CD5 and IgM clonal expression. (Fig 4)

While no significant associations were detected between hypoalbuminemia and LDH levels. (Fig 5, 6)

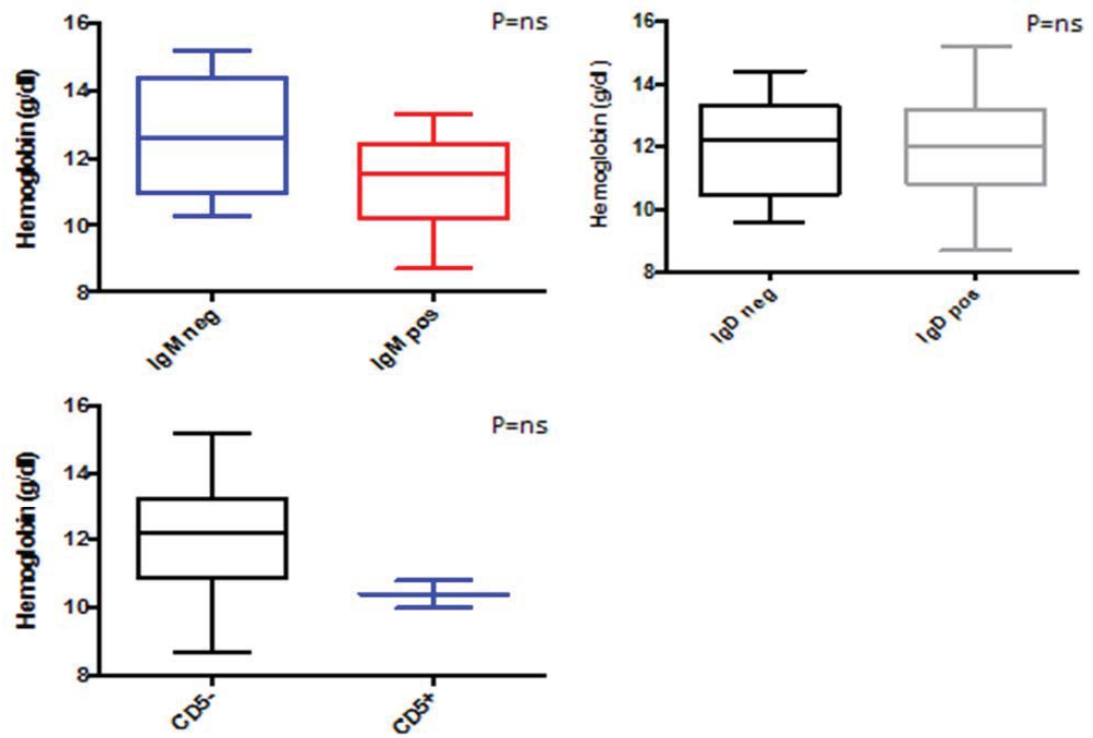


Fig 4 Correlation between low Hemoglobin concentration and CD5 and IgM clonal expression

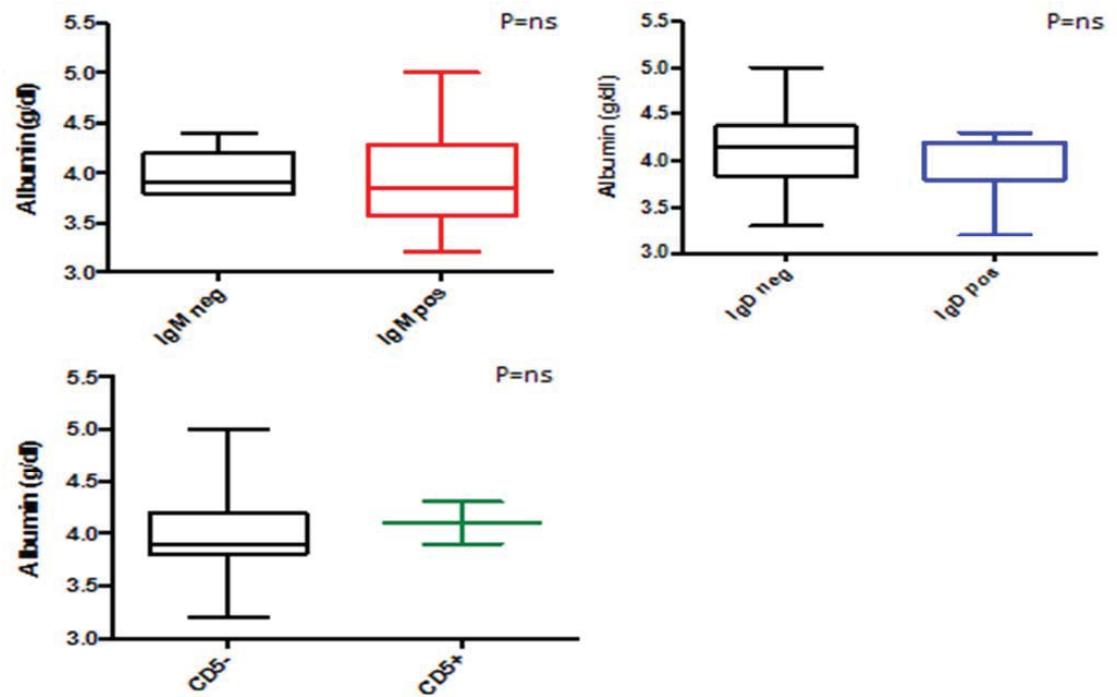


Fig 5 Correlation between albumin concentration and CD5 and IgM clonal expression

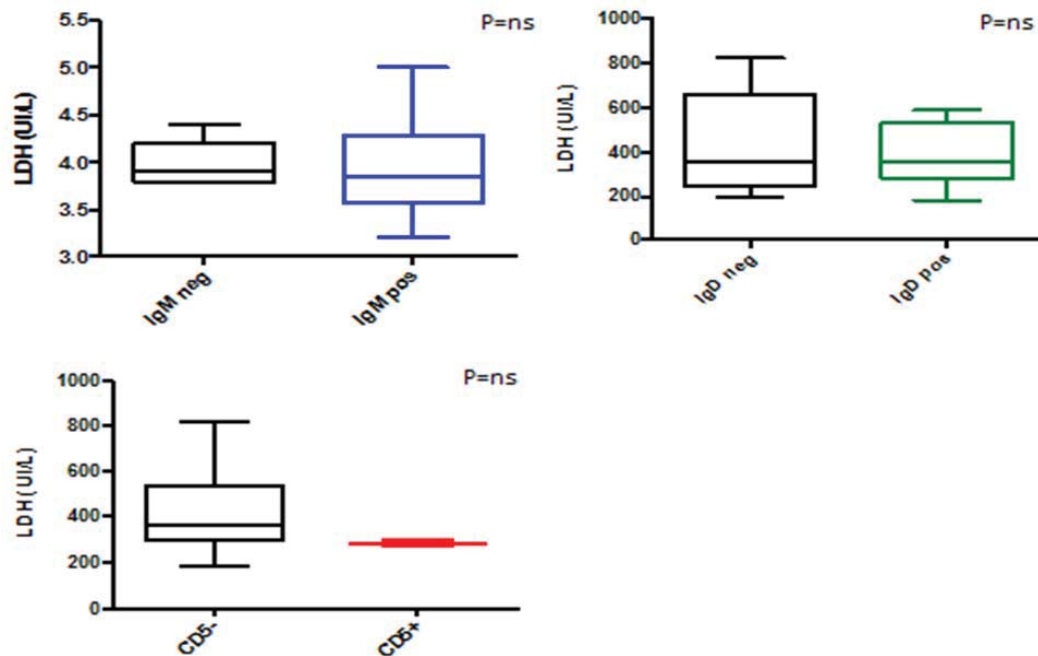


Fig 6 Correlation between LDH concentration and CD5 and IgM clonal expression.

As regarding the risk category, the analysis highlighted the correlation between CD5 and IgM expression and the intermediate/high risk categories. (Fig 7)

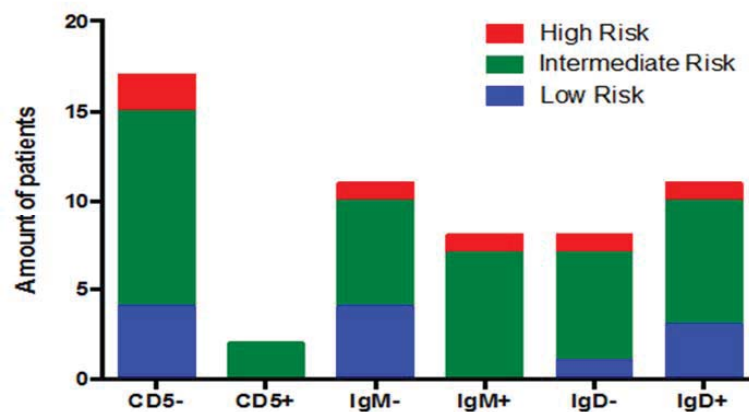


Fig 7 Correlation between CD5 and IgM expression and the intermediate/high risk categories.



On the base of these results, we can speculate that CD5 and/or IgM expression may be considered as unfavorable prognostic markers to progression disease.

As already known in the literature, B-cell neoplastic clone displays a relevant tropism for the vascular bone marrow niche.

We found that, although it is well integrated in the vascular sinusoidal compartment (Fig 8), in few cases it forms pseudo-nodular aggregates, constituted by follicular dendritic cells (FDC), expressing both CD23 and vimentin and generally present within non-Germinal Center neoplastic infiltrates (Fig 9)

The preexisting stroma may be remodeled by the clone and this feature is representative of cases of SMZL in progression toward high-grade lymphoma.

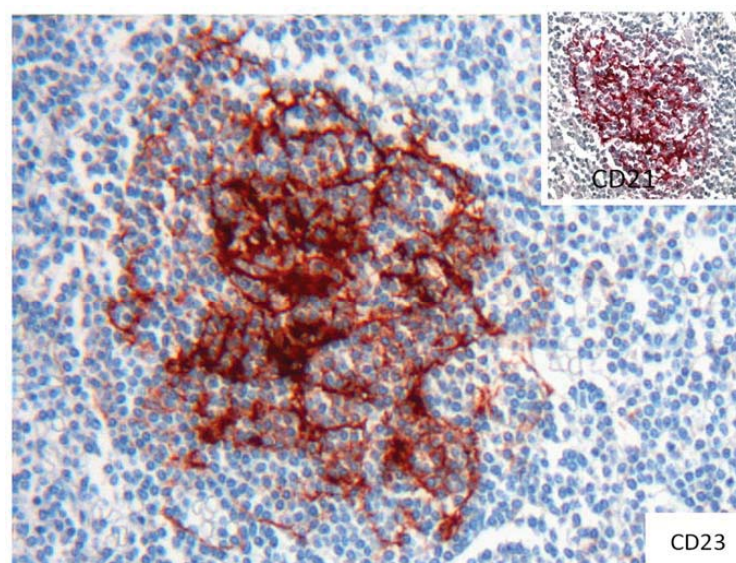


Fig 8 FDC network (CD23+; CD21+) in SMZL

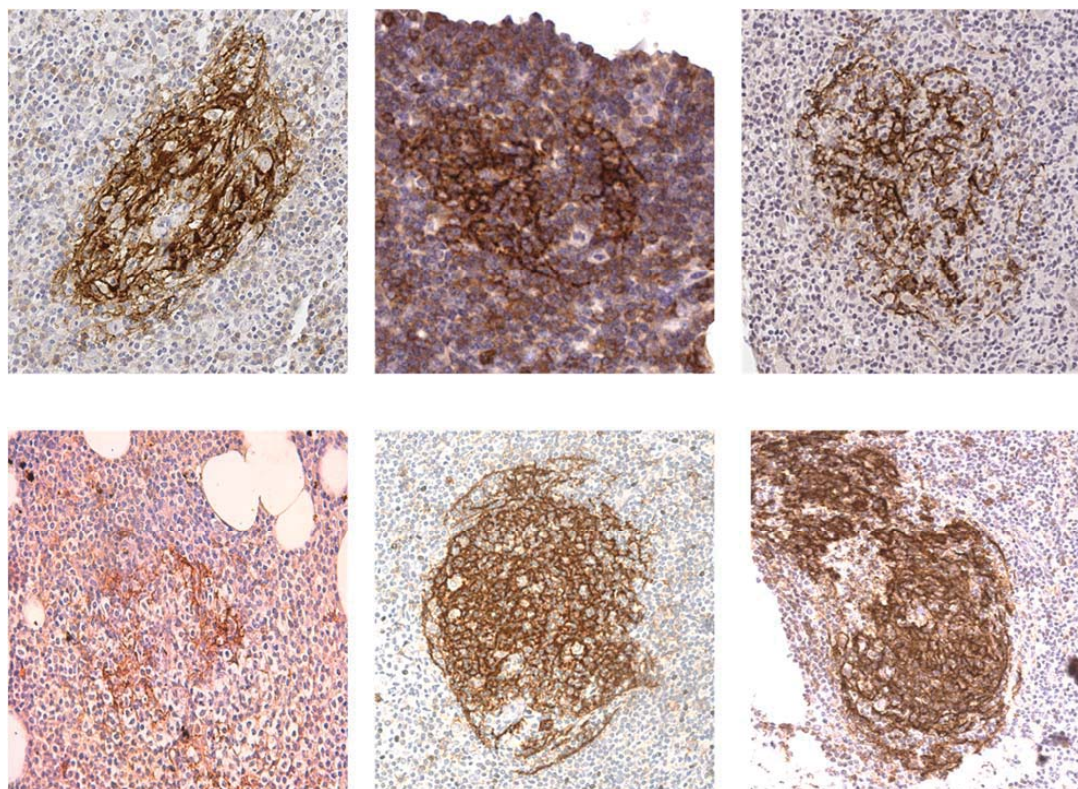


Fig 9 Germinal Centre-like structures with FDC network (CD23+)

Keeping focus on stromal microenvironment characterizing germinal center, we have dissected the role of extracellular matrix protein such as osteopontin in tumours and in particular in B-cell malignancies.

Osteopontin (OPN) is a pleiotropic cytokine initially described as early T lymphocyte activation-1 (ETA-1) <sup>(72)</sup> also acting as a matricellular protein that shares with SPARC some receptors, such as integrins.

Moreover, polymorphisms of the gene *Spp1* (encoding OPN) are present in autoimmune mice and humans.

In cancer, OPN has been implicated in the tumor-stroma interface, especially at the metastatic niche, in which it plays its role also through the direct regulation of the suppressive immune microenvironment.

OPN is expressed in GC microenvironment in human lymphoid tissues either with reactive conditions or prototypical GC driven malignant lymphomas. (Fig 10)

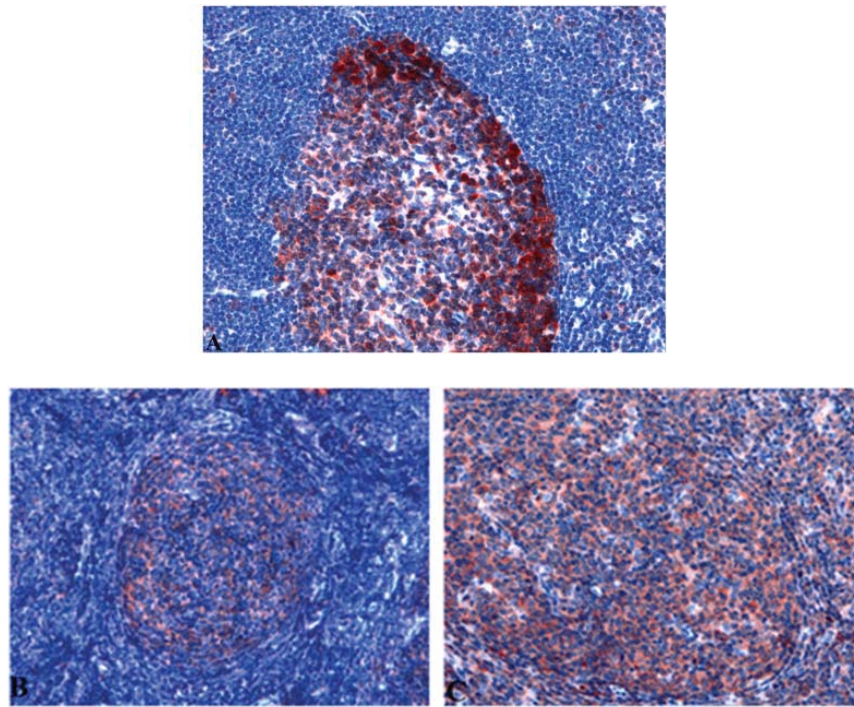


Figure 10 OPN expression in follicular hyperplasia (A) and in follicular lymphoma G1/G2 (B) and G3 (C)

Among NHLs, SMZL is associated with dysregulated immune stimulation, which in almost one-third of patients displays an aggressive course and evolve toward diffuse large B-cell lymphoma (DLBCL).

As a model of dysregulated immune stimulation we adopted Fas<sup>lpr/lpr</sup> mice, which develop a Lupus-like autoimmune disease owing to Fas mutation.

In autoimmune Fas<sup>lpr/lpr</sup> mice, a murine model of SLE, disarranged secondary lymphoid organ (SLO) architecture, due to deficiency in the matricellular protein SPARC, caused early autoimmunity degenerating into a CD5<sup>+</sup> B-CLL-like lymphoma. <sup>(73)</sup>

In Fas mutant mice, one of the cytokines that are upregulated early upon secondary lymphoid organ remodelling is OPN. <sup>(73)</sup>

To test the role of OPN to autoimmunity-driven lymphomagenesis, Fas<sup>lpr/lpr</sup> mutation



has been transferred into the OPN-deficient background. *Fas* gene mutation (*Fas*<sup>lpr/lpr</sup> mice), when associated with osteopontin (OPN) deficiency led to an exacerbated disease and to enhanced B cell proliferation, which, in aged mice, evolved towards the development of diffuse large B-cell lymphomas with phenotypic and molecular features of the activated B-cell type (ABC-DLBCL). (Fig 11)

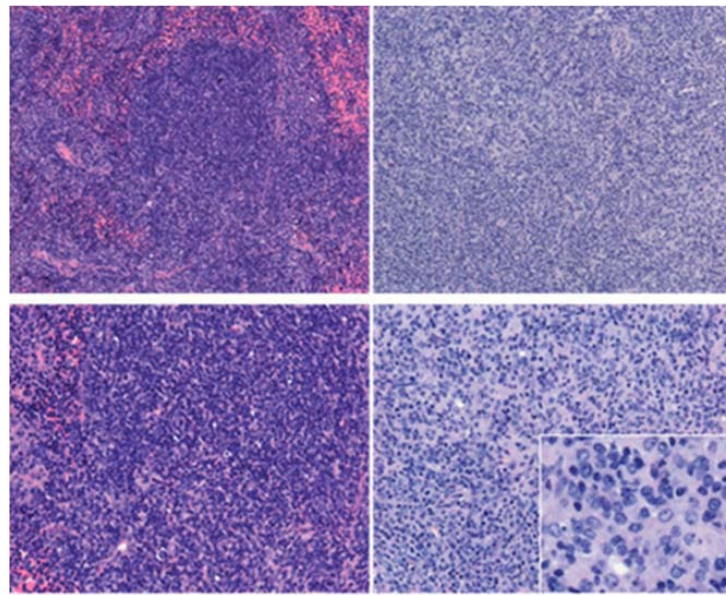


Fig 11 Exarcebated autoimmunity and DLBCL-like lymphoproliferation in *Fas*<sup>lpr/lpr</sup>*OPN*<sup>-/-</sup>

This murine model allowed to investigate how B-cell intrinsic defects accumulate along discrete stages of lymphomagenesis and OPN deficiency in tumor-associated stromal microenvironment provide the opportunity to understand the pathogenesis of DLBCL and to identify novel tumour or microenvironment targets of therapeutic interests for the treatment of DLBCL.

Taking advantage from a model of spontaneous lymphomagenesis and investigating the mechanism regulating the development of high grade lymphoma, we highlighted the interplay between B-cell-intrinsic and microenviromental factors that might correlate within human lymphomas, eventually associated with autoimmune conditions.

Finally, to evaluate the influence of microenvironment in DLBCL, we investigated the stromal and immune tumour- associated microenvironment in the two subtypes of this lymphoma, GC and ABC.

The stromal composition and the quality of immune infiltrate are functionally linked to the histotype although heterogeneous. In fact, trends can be envisaged for GC and non-GC groups. (Fig 12-14)

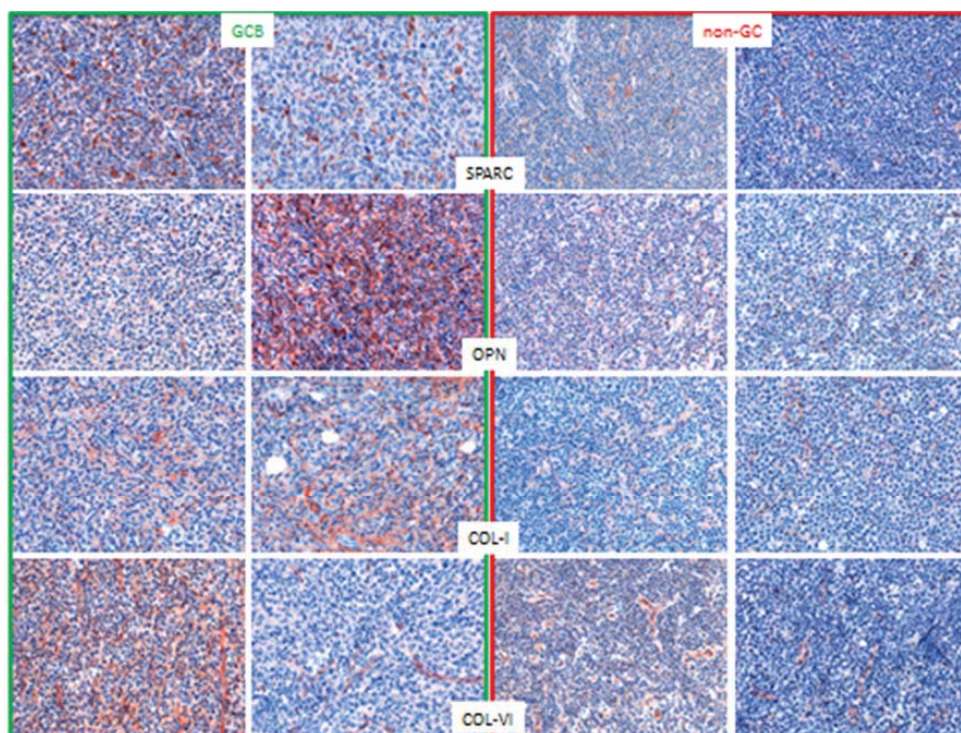


Fig 12 Differences of the stromal composition of microenvironment between GCB and non GC DLBCL by IHC staining.



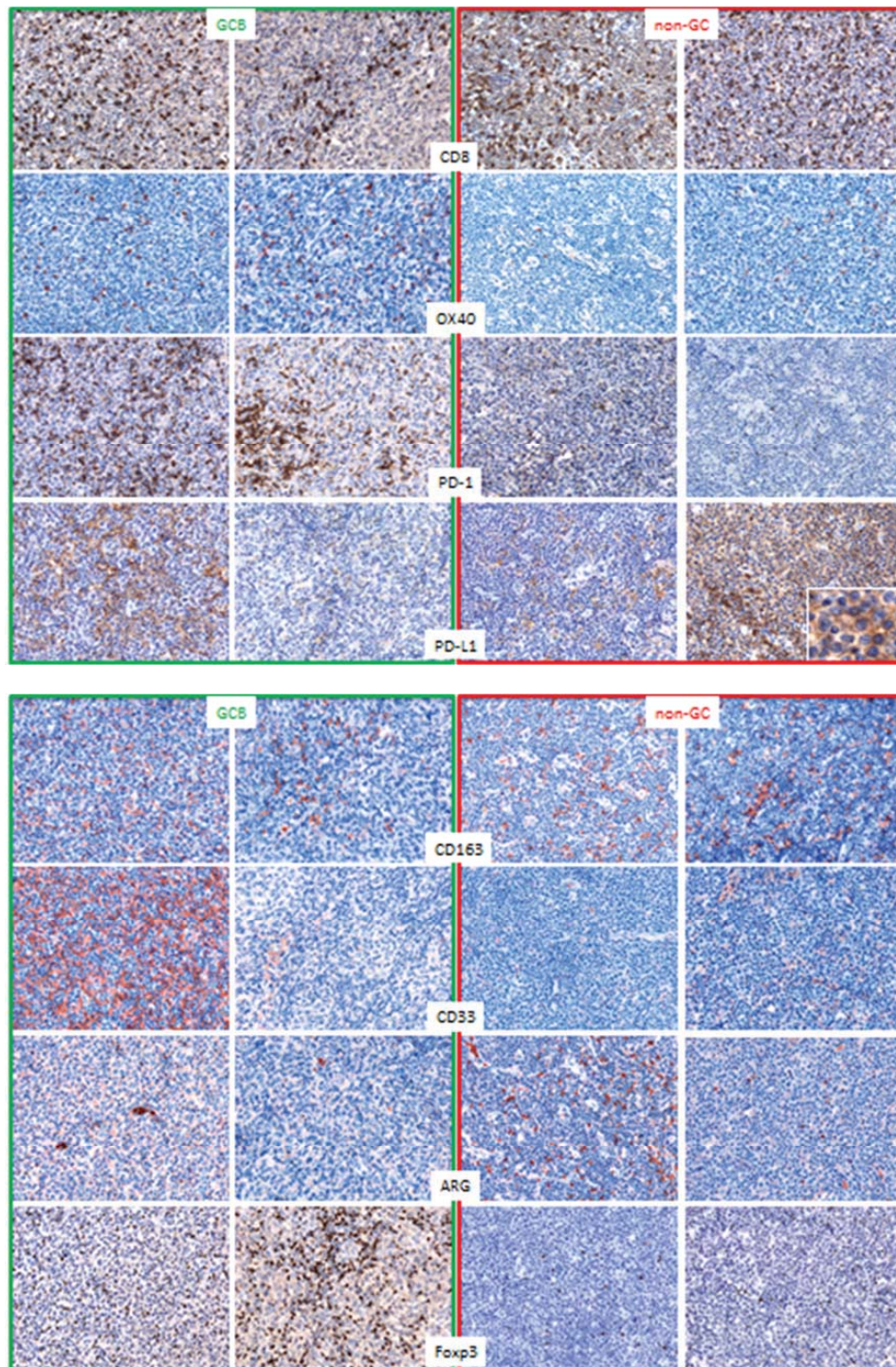


Fig 13, 14. Variable distribution of immune cell infiltrate in GC and non GC DLBCL by IHC staining.

Our data suggest that the presence and eventually distribution of stromal and immune

cell infiltrate within microenvironment in GC and non-GC DLBCL may be considered as therapeutic targets and may also have a predictive value for outcome of this patients. Furthermore, other immunohistochemical studies have demonstrated a correlation between the stromal and immunological microenvironment and outcome in the two subtypes.

In the subtype with good prognosis it is detected the presence of FDC (S100+/CD21+) together with dense infiltrate of Cytotoxic T Lymphocytes(CTL), T-Reg (FOXP3+) and mast cells. Contrary, poor prognostic subtype is characterized by dense cytotoxic T lymph infiltrates. <sup>(74-79)</sup>

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