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PhD in IMMUNOLOGICAL SCIENCE
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"Immunophenotipic and molecular characterization of EBV-driven age-related lymphoproliferative disorders"

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INTRODUCTION

1.1 EPSTEIN BARR VIRUS-INDUCED LYMPHOMAGENESIS

Epstein Barr Virus (EBV) is a member of the herpes virus family that infects more than 90% of the wordwide population (*Rickinson et al, 2006*). In developing countries, primary lytic infection occurs in the oropharynx in early childhood usually asymptomatically, while, in developed countries of high socio-economic status it is delayed until adolescence and one third of the cases results in infectious mononucleosis (*Rickinson et al, 2006*; *Henle et* al*, 1968*). In the oropharynx, EBV infects via the CD21 (CR2) receptor epithelial cells lining the inner mucosal surfaces of the mouth and nose, and naïve B cells circulating through mucosal site. EBV is highly immunogenic and it induces a vigorous immune response so that, soon after primary infection, EBV persists as an episome in a small proportion of infected B cells, establishing latent infection (*Niedobitek et al, 1997*). There are 4 different types of latency, each characterized by a specific pattern of EBV antigen expression (Figure 1) (*Heslop,* 2009). Most circulating infected memory B cells down-regulate viral antigens (type 0 latency) and are invisible to the immune system. In type 1 latency the nuclear protein EBNA-1 is expressed in proliferating B cells where it induces replication of the viral episome. Type 2 latency is associated with the expression of EBNA-1, and of the membrane proteins LMP-1 and LMP-2 in germinal center B cells. In type 3 latency infected B cells expressed all nuclear proteins (EBNA-1, -2, -3A, 3B, 3C, and LP), LMP-1 and LMP-2, and 2 small RNAs (EBERs). Type 3 latency is the only type able to transform primary B cells *in vitro*; however, it is also the most immunogenic, so that it is rarely detected in healthy persons probably due to a rapid and efficient EBV-specific T-cell response (*Rowe et al, 1992; Ma et al, 2011*).

In has been shown that *in vitro* EBV infects resting B cells and transform them through complex mechanisms into permanently growing lymphoblastoid cell lines. This property makes it a candidate causative for many human cancers including epithelial and haematopoietic tumors (*Rickinson et al, 2006*). Although the true contribution of EBV to the lymphomagenesis remains to be elucidated, it is thought that EBV may unable EBV-infected B cells to exit the cell cycle to become a resting memory B-cell, resulting in their immortalization and continuous proliferation (*Sugden et al, 1989; Saha et al, 2011*) EBNA-1 is a master transcriptor factor that is required for immortalization; it upregulates p53 expression levels and a variety of other latency genes, as well as host activation proteins (*Yates et al,* 1984; *Saridakis*

et al, 2005). EBNA-2 has been suggested to be involved in G0 to G1 phase transition however, although it appears essential for initial growth transformation of infected B cells *in vitro*, it seems to have a secondary role in tumor progression (*Johannsen et al*, 1995). EBNA-3A and -3C are also essential for B-cell immortalization acting through different pathways (Table 1) (*Anderton et al*, 2008; *White et al*, 2010). Among EBV-encoded proteins, LMP-1 is the major transforming one; it has been shown to act as an oncogene in rodent fibroblast transformation assay (*Wang et al, 1985*; *Kaye et al, 1993*). There are evidences that LMP1 acts as a constitutively active receptor; in particular, LMP-1 mimics activated CD40, a member of the tumor necrosis factor receptor family, stimulating growth and differentiation responses in B cells (*Uchida et al, 1999*). Moreover, LMP-1 activates the nuclear factor (NF)-kB signalling cascade (*Zhimin et al, 2000*), and up-regulates the expression of the anti-apoptotic proteins Bcl-2 and A20 stimulating B-cell growth (*Kulwichit et al 1998; D'Souza et al,2004).*

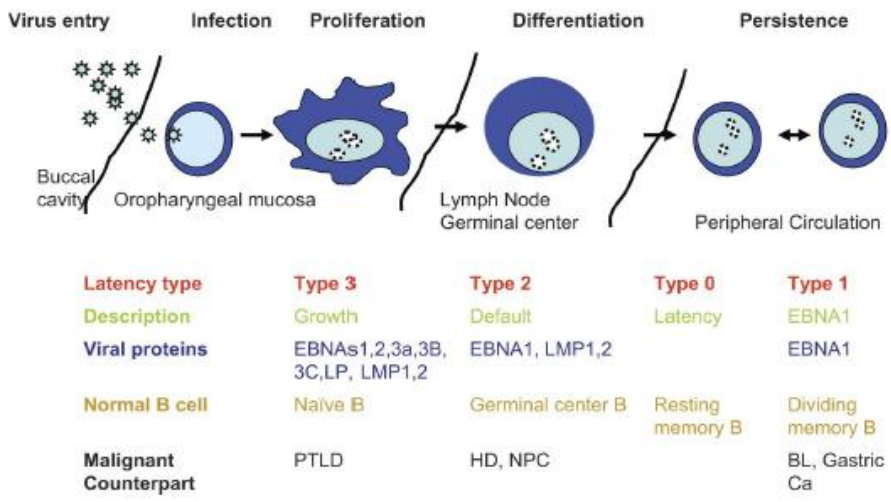

Figure 1. EBV latent life cycle. Virus enters though mucosal routes (shown is the buccal cavity), then infects normal naive B cells circulating through mucosal sites. Virus expresses type 3 latency, which drives B-cell proliferation and expands the infected memory pool. B-cell differentiation into the memory compartments occurs in germinal centers driven by type 2 latency proteins. Infected memory B cells exiting the germinal center down-regulate viral proteins and are invisible to the immune response. EBNA1 is expressed during homeostatic proliferation to maintain the latent viral episome. Virus replication is induced at mucosal sites, and virus is released into the saliva. PTLD indicates posttransplantation lymphoproliferative disease; HD, Hodgkin disease; NPC, nasopharyngeal cancer, and BL, Burkitt lymphoma.

(Heslop HE. Blood. 2009 Nov 5;114(19):4002-8.)

An impairment in T-cell response against infected B-cells seems to be crucial for the expansion of transformed cells (*Thorley-Lawson et al, 2004; Hislop et al, 2007*). Immune control of EBV is mediated primarily by T cells; in particular, while $CD8^+$ T cells target primarily the EBNA3 and LMP2 proteins, $CD4^+$ T cells most frequently react to EBNA1 (*Murray et al 1992; Khanna et al, 1992; Munz et al, 2000; Leen et al, 2000*). Recently, White et al suggested that loss of EBNA3B function in tumor cells

through *EBNA3B* gene mutation cause substantial changes in the character, immune evasion, and aggressiveness of EBV-associated cancer. In this context, escape from HLA-A11–restricted recognition of EBNA3B was previously suggested as the cause of the uncontrolled growth of a post-transplant DLBCL (*White et al, 2012*).

Table 1. Proposed Functions of EBV Latent Antigens and the Associated B-cell Lymphomas

Latent proteins	Functions	Associated lymphomas	Latency type
EBNA-1	Essential for B-cell immortalization, replicates EBV genome, segregates viral episomes at mitosis, blocks interaction between HAUSP and p53 to facilitate p53's degradation	BL Hodgkin's disease AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	I, II, III
EBNA-2	Transcriptional coactivator that upregulates expression of viral (LMP1) and cellular genes (c-*myc*), essential for B-cell immortalization	AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	III
EBNA-3A	Essential for B-cell immortalization of cell, interacts with RBP-Jκ, regulates Notch-signaling pathway	AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	III
EBNA-3B	Not essential for B-cell immortalization, interacts with RBP-Jκ	AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	III
EBNA-3C	Essential for B-cell immortalization, overcomes various checkpoints in cell cycle, interacts with RBP-Jκ, activates LMP1, blocks p53-dependent apoptosis, enhances kinase activity of both cyclin D1/CDK6 and cyclin A/CDK2 complexes, induces degradation $p27^{KIP1}$ and pRb, stabilizes c-Myc, Cyclin D1, and Mdm2, manipulates host chromatin remodeling machinery	AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	III
EBNA-LP	Interacts with EBNA2 to inactivate p53 and pRb, interacts with transcription factors in notch signaling pathway, contributes to B-cell immortalization	AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	III
LMP-1	Mimics CD40 ligand-binding signal, stimulates bcl-2 and a20 expression to block apoptosis, acts as a constitutively active receptor for stimulating many cellular genes, regulates NF-κB, JAK/STAT, ERK MAPK, IRF, and Wnt signaling pathways, essential for B-cell immortalization	Hodgkin's disease AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	II, III
LMP-2A and -2B	Drives EBV into latency, LMP-2A blocks BCR signaling and -2B assists its function, not essential for B-cell immortalization	Hodgkin's disease AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	II, III
EBERs	Form complexes with La and L22, associate with PKR, induce IFN and IL-10, bind to RIG-1 to activate type I IFNs, not essential for B-cell immortalization	BL Hodgkin's disease AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	I, II, III
BARFs	Protein products may modify Notch signaling, not essential for B-cell immortalization	BL Hodgkin's disease AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	I, II, III

(Saha A. et al. Clin.Cancer Res. 2011 May15;17 (10):3056-3063)

Alternatively, the disruption of the normal balance between latently infected B-cell proliferation and the EBV-cytotoxic T-cell response could be due to an immunodeficient status of the patient or to the administration of immunosuppressive agents (e.g. for organ transplantation). In support of this view, is the frequent evidence of EBV infection in

neoplastic cells of B-cell lymphoproliferative disorders (LPD) occurring in patients with primary immune deficiency, HIV infection, or with iatrogenic immunosuppression (*Swerdlow et al, 2008*).

1.2 EBV-ASSOCIATED LYMPHOPROLIFERATIVE DISORDERS

Since its discovery as the first human tumorigenic virus, EBV has been implicated in the development of a wide range of B-cell (Table 2) and of T/NK-cell lymphoproliferative disorders (Table 3). EBV-associated malignancies arise in both immunosuppressed and immunocompetent individuals, and involve the expression of either some or all of the EBV latent proteins (Figure 1).

1.2.1 EBV-ASSOCIATED B-CELL LYMPHOPROLIFERATIVE DISORDERS

Despite its documented infection in T lymphocytes and epithelial cells, EBV has a major preference for B cells, and under certain circumstances the infected B cells can transform into malignant B-cell lymphomas (*Bajaj et al, 2007*). They include: Hodgkin lymphoma, Burkitt lymphoma, post-transplant lymphoproliferative disorders, lymphomatoid granulomatosis, age-related EBV-associated B-cell lymphoproliferative disorders, diffuse large B cell lymphoma associated with chronic inflammation, and many B-cell lymphomas associated with HIV-infection (*Swerdlow et al, 2008; Carbone et al, 2008*). Different types of EBV-latency are associated with these lymphomas (Figure 1).

Burkitt's Lymphoma

Burkitt's lymphoma (BL) is an aggressive B-cell tumor, often presents in extranodal sites or as an acute leukemia, characterized by the expansion of medium-sized cells with a very high proliferation index (>98%). It includes three variants: endemic (mainly in children from equatorial Africa, Papua, and New Guinea), sporadic (affecting children and young adults throughout the world), and immunodeficiency-related (mostly associated with HIV-infection). EBV has been detected in virtually all cases of the endemic variant, 20%–30% of the cases of the sporadic variant, and 30%–40% of cases of the immunodeficiency-related variant (*Carbone et al, 2008*). In all EBV-positive cases, EBV is found in the majority of the neoplastic cells with a type I latency pattern given by the expression of EBNA-1 and EBERs only (*Weiss et al, 2012*). In endemic Burkitt lymphoma the strong epidemiological link with holoendemic malaria suggests a polymicrobial pathogenesis (*Rochford et al, 2005*). By this model, infection

with Plasmodium Falciparum could reactivate latently infected B cells through toll like receptor 9 (TLR-9) and, at the same time, impact on immunity exhausting EBV-specific T cell response. In all variants, irrespective of EBV status, translocation of the c-myc oncogene into one of the immunoglobulin loci is undoubtedly the key factor in the oncogenesis of Burkitt's lymphoma (*Della Favera et al, 1982; Taub et al, 1982; Hummel et al, 2006*). There is some evidence that EBV-BL arises from a latency pattern-3 progenitor that under a selection pressure down–regulates the c-myc antagonist EBNA-2 (*Weiss et al, 2012*). The detection of somatic hypermutations in the V region of clonally rearranged immunoglobulin genes and the phenotype of the lymphoma cells indicate a germinal center (GC) cell origin of the Burkitt lymphoma (*Chapman et al, 1996*).

Table 2. EBV-associated lymphoproliferative disorders

EBV-associated B-cell lymphoproliferative disorders
Burkitt's lymphoma
Classic Hodgkin's lymphoma
Post-transplant lymphoproliferative disorders
HIV-associated lymphoproliferative disorders
Primary central nervous system lymphoma
Diffuse large B-cell lymphoma, immunoblastic
HHV-8–positive primary effusion lymphoma and its solid variant
Plasmablastic lymphoma
Other histotypes (rare)[a]

[a]Other histotypes include: lymphomatoid granulomatosis, pyothorax-associated lymphoma, senile EBV-associated B-cell lymphoproliferative disorders.
Abbreviations: EBV, Epstein–Barr virus; HHV-8, human herpesvirus 8.

(*Carbone A et al. Oncologist. 2008 May;13(5)*:577-85.)

EBV-Associated Lymphomas in Immunocompromised Individuals

There exist several distinct EBV-associated lymphoproliferative disorders (LPD) in immunocompromised individuals. Some LPD are associated with primary immune disorders such as X-linked lymphoproliferative syndrome (XLP), severe combined immunodeficiency (SCID), hyper-IgM syndrome, common variable immunodeficiency (CVID), Wiskott-Aldrich syndrome (WAS), while others are associated with immunosuppressive drugs given to transplant recipients (PTLDs) or with HIV-infection. Finally, there are lymphoproliferative

disorders that arise in patients treated with immunosuppressive drugs for autoimmune diseases or for conditions other than in the transplant setting (*Swerdlow et al, 2008*). The most common EBV gene-expression pattern in these disorders is latency III (Figure 1).

Post transplant lymphoproliferative disorders (PTLDs)

PTLDs are lymphoproliferative disorders that develop as a consequence of immunosuppression in transplant recipients of both a solid organ and bone marrow. According to the World Health Organization classification (*Swerdlow et al, 2008*), PTLDs may be classified into: (a) early lesions, generally represented by EBV-driven polyclonal or oligoclonal lymphoproliferations, and (b) true monoclonal diseases, that are not necessarily associated with EBV infection, including polymorphic PTLDs and monomorphic PTLDs. The latter are indistinguishable from those that occur in immunocompetent individuals and, are further distinguished into Burkitt's lymphoma/Burkitt's-like lymphoma, diffuse large B-cell lymphoma (DLBCL), and cHL.

Oncogenic viruses known to be involved in PTLD pathogenesis include EBV and human herpes virus 8 (HHV-8). Several lines of evidence suggest that EBV infection has a major pathogenetic role in PTLDs: (a) EBV infects 70%– 80% PTLD patients, including 100% of early-onset PTLD cases; (b) in many monomorphic PTLD cases, EBV infection is monoclonal, suggesting that the virus might be present in the tumor progenitor cell since the early phases of clonal expansion; (c) an increase in the EBV viral load and a decrease in the number of EBV-specific cytotoxic T lymphocytes (CTLs) have found to be strongly associated with PTLD development; and (d) treatment of PTLDs with autologous EBV-specific CTLs results in viral load control and tumor size reduction (*Davis et al, 2004*; *Carbone et al, 2008*). Up to 30% of PTLD are EBV-negative, and although some can be driven by HHV-8, others can still be triggered by a no longer detectable EBV or, alternatively, by other unknown viruses (*Swerdlow et al, 2008*).

Other iatrogenic immunodeficiency-associated lymphoproliferative disorders

Other iatrogenic LPD can occur in patients treated with immunosuppressive drugs for autoimmune diseases. It is actually difficult to determine how many LPD are directly related to

the iatrogenic immunosuppression rather than the underlying disorder, however, it is likely that risk and type of LPD depend on the type of immunosuppressive agent (e.g. methotrexate, infliximab, TNF blocks) and on the nature of the underlying disorder (e.g. rheumatoid arthritis, inflammatory bowel disease, dermatomyositis, psoriasis). Most of the cases are diffuse large B cell lymphomas, while others are more polymorphic or resemble Hodgkin lymphoma. In half of the cases the localization is extranodal, and about 50% of the cases are EBV-positive (*Au et al, 2006*; *Swerdlow et al, 2008*).

HIV-Associated Lymphoproliferative Disorders

HIV-associated lymphoproliferative disorders are a heterogeneous group of diseases that arise in the presence of HIV-associated immunosuppression, a state that permits the unchecked proliferation of EBV-infected lymphocytes. Traditionally, these aggressive disorders include both central nervous system and systemic lymphomas. Pleural effusion lymphomas (PEL) also occurs and often involves EBV in addition to HHV-8 (*Carbone et al, 2008*). The categories of HIV-associated lymphomas included in the latest WHO classification of tumors of haematopoietic and lymphoid tissues are grouped as follows: (a) lymphomas also occurring in immunocompetent patients such as: Burkitt's lymphoma (30% EBV+), systemic DLBCL with centroblastic features (30% EBV+), and DLBCL with immunoblastic features frequently involving the central nervous system (100% EBV+); (b) unusual lymphomas occurring more specifically in HIV-positive patients, including: PEL (80% EBV+ in addition to 100% HHV8+) and plasmablastic lymphoma of the oral cavity (60-70% of the oral cavity; (c) lymphomas also occurring in other immunodeficiency states *(Swerdlow et al, 2008)*.

EBV+ diffuse large B cell lymphoma of the elderly

EBV+ diffuse large B cell lymphoma of the elderly is an EBV+ lymphoma occurring in patients over 50 years with no other cause of immunodeficiency. It represents up to 10% of elderly lymphomas in Asian population and about 4% of DLBCL in Western countries (*Gibson et al, 2009; Hoeller et al, 2010*). Its incidence increases with increasing age, and is slightly predominant in male. Most patients (70%) have an extranodal disease, including skin, lung, tonsil, and stomach. The disease is clinically aggressive with a poor prognosis.

Morphologically, it is subclassified in polymorphic and monomorphic. The polymorphic subtype show a proliferation of B cells with a broad range of maturation, whereas in the mononorphic variant neoplastic B cells have either centroblastic or immunoblastic features. In both subtypes are present Hodgkin and Reed Stenberg(H/RS)-like cells. EBER is positive in the neoplastic cells, whereas LMP-1 may be negative. The EBV latency pattern is of type III. It is believed that the development of this lymphoma is related to immunosenescence that is part of the aging process (*Swerdlow et al, 2008*).

Diffuse large B cell lymphoma (DLBCL) associated with chronic inflammation

The disease arise in the context of local long-standing inflammation, usually after decades after the onset of the inflammation. Therefore, patients are usually old with a male predominance. Most cases are reported to be associated with pyothorax occurring many years after artificial pneumothorax for pulmonary tubercolosis. Also, it has been described in bones of patients with chronic osteomyelitis, joints, and soft tissues. It is an aggressive lymphoma with about 25% of 5-year overall survival. Histologically, it resembles EBV+ diffuse large B cell lymphoma of the elderly, and as for it, EBV latency pattern is type III in more than 60% of the cases (*Swerdlow et al, 2008*). It is supposed that local chronic inflammation may favour immune escape of EBV-transformed B cells trough the production of large amount of IL-10, an immunosuppressive cytokine, and that it may also provide autocrine to paracrine tumor growth via the production of IL6 (*Kanno et al, 1996; Kanno et al, 1997*).

Lymphomatoid granulomatosis

Lymphomatoid granulomatosis is a rare angiocentric and angiodestructive lymphoproliferative disease involving extranodal sites (i.e. lung, skin, kidney, brain and, liver). It often develops in patients with an underlying immunodeficiency (e.g. post-organ transplantation therapy, Wilskott-Aldrich syndrome, HIV-infection, and others). Morphologically, It is composed of a small number of large EBV-positive B cells associated with numerous small reactive T cells (*Swerdlow et al, 2008*).

1.2.2 EBV-ASSOCIATED T/NK-CELL LYMPHOPROLIFERATIVE DISORDERS

T-cell lymphoproliferative disorders that have been reported to be EBV associated include a subset of peripheral T-cell lymphomas (PTLC), angioimmunoblastic T-cell lymphoma (AILT), extranodal nasal type NK/T-cell lymphoma, enteropathy-type T-cell lymphoma, hepatosplenic T-cell lymphoma, systemic EBV+ T-cell Lymphoproliferative disease of childhood, Hydroa vacciniforme-like lymphoma, and aggressive NK-cell leukemia/lymphoma. However, many T cell lymphoma may contain scattered EBV+ cells as a reflection of the generalized immunodeficiency that is sometimes part of a peripheral T-cell lymphoma (*Carbone et al, 2008*; *Swerdlow et al 2008*).

Table 3. EBV-associated lymphoproliferative disorders

EBV-associated T/NK-cell lymphoproliferative disorders
Peripheral T-cell lymphoma, unspecified
Angioimmunoblastic T-cell lymphoma
Extranodal nasal T/NK-cell lymphoma
Other histotypes (rare)[a]

[a]Other histotypes include hepatosplenic T-cell lymphoma, nonhepatosplenic γδ T-cell lymphomas, enteropathy-type T-cell lymphoma.
Abbreviations: EBV, Epstein–Barr virus; NK, natural killer.

(Carbone A et al. Oncologist. 2008 May;13(5):577-85.)

Angioimmunoblastic T cell lymphoma (AILT)

AILT is one of the most common PTCL subtype. It is characterized by systemic disease, a polymorphous infiltrate primarily involving lymph nodes, and prominent proliferation of high endothelial venules and follicular dendritic cells (*Swerdlow et al 2008*). The clinical behavior is very aggressive with a scarce response to therapy. The molecular pathogenesis of AILT, as in general for all peripheral T-cell neoplasms, is poorly understood. Characteristically, AILT is a lymphoma in which expanding B-cell clones are often present beside the T-cell clones. AILT is also associated with EBV in a high proportion of cases (97%), but in this lymphoma, the EBV is seen mainly in the B lymphocytes and in large atypical H/RS-like B-cells (*Zettl et al*,

2002; Quintanilla-Martinez et al, 1999; Smuk et al, 2010). The presence of EBV in only a subpopulation of cells suggests that EBV infection is not part of the pathogenesis of the disease, but a manifestation of the diminished local immune surveillance or alternatively, that the viral genome has been lost from the malignant T cells.

Extranodal NK/T-cell lymphoma nasal type

Extranodal NK/T-cell lymphoma nasal type is a predominantly extranodal lymphoma characterized by vascular damage by a population of neoplastic cells characterized by absence of T-cell antigens and by expression of the NK cell marker CD56. Clinically, these tumors occur in the nasal and upper aerodigestive track, and it is more common in Asian and in native Americans of Central and South America. EBV is consistently associated with these lymphomas, regardless of geographical location. Tumor cells are always positive for EBER, and mostly negative for LMPI-1 (*Swerdlow et al 2008; Weiss et al 2012*).

Systemic EBV+ T cell lymphoproliferative disease (LPD)

Systemic EBV+ T-cell LPD is a recently recognized clonal proliferation of EBV-infected T cells with an activated cytotoxic phenotype (TIA-1+). It occurs mainly in children and young adults frequently from Asia and Mexico. It can occur shortly after primary acute EBV infection or in the setting of chronic active EBV infection (CAEBV). It is a systemic disease with involvement of liver, spleen, lymph nodes, bone marrow, skin, and lungs. The clinical course is very aggressive with death occurring just after days or weeks. EBER is consistently positive in neoplastic CD8+ T cells. Its association with primary EBV infection strongly suggest the presence of a genetic defect in the host immune response to EBV that may predispose to EBV+ T cell LPD (*Swerdlow et al 2008; Weiss et al 2012*).

Hydroa vacciniforme like- lymphoma

It is an EBV+ cutaneous T-cell lymphoma occurring in children and adolescence and associated with hypersensitivity to insect bites and sun. It is seen mainly in Asian and in native

Americans of Central and South America. It involves sun-exposed skin with papulo-vescicular lesions that ulcerates. Clinical course is variable, it may be indolent for several years, or may consists in recurrent skin lesions before progression to systemic involvement. Neoplastic T-cells consistently express EBER, whereas LMP-1 is generally negative(*Swerdlow et al 2008*).

1.3 HODGKIN LYMPHOMA AND EBV

1.3.1 Classification and morphology

Based on differences in histological characteristics, cell of origin, clinical features, and molecular pathogenesis HL is categorized in 2 distinct entities: nodular lymphocyte predominant lymphoma (NLPHL), and classical Hodgkin Lymphoma (cHL).The latter is further subclassified into 4 variants: nodular sclerosis (NS), mixed cellularity (MC), lymphocyte-rich (LR), and lymphocyte-depleted (LD) (*Swerdlow et al 2008*).

A striking feature of both cHL and NLPHL is that in the affected lymph node malignant cells are very rare (0.1-2% of tumor mass) and are scattered in a background rich in small lymphocytes, plasma cells, histiocytes and eosinophils (Figure 2). In cHL the neoplastic cells are referred to as Hodgkin and Reed Sternberg (H/RS) cells. Hodgkin cells are large and mononucleated with prominent eosinophilic nucleoli, whereas Reed Sternberg cells are bi- or multinucleated (Figure 2A). In NLPHL tumor cells are indicated as lymphocyte predominant (LP) cells (formerly called L&H cells) and show a multilobated morphology generally lacking evident nucleoli ("popcorn" cells) (Figure 2B) (*Swerdlow et al 2008*).

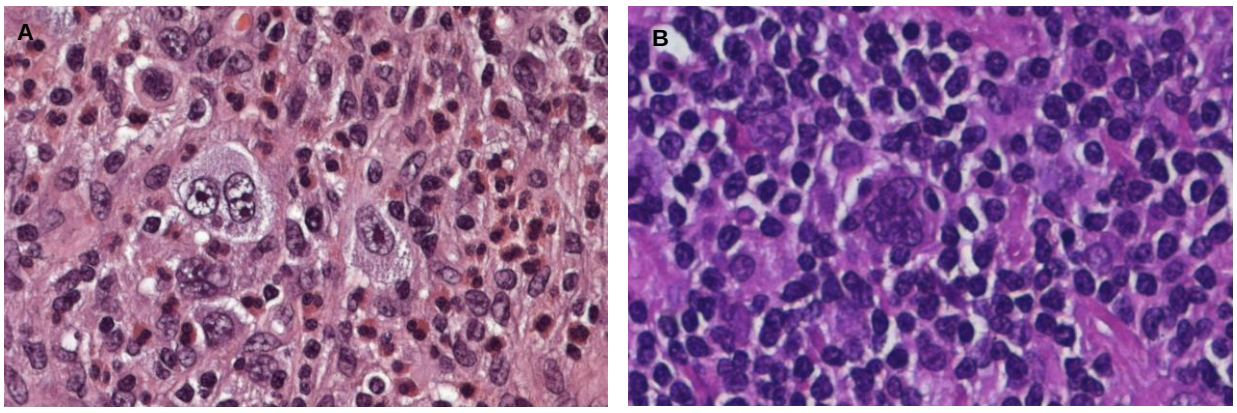

Figure 2. **A**. Hodgkin and Reed Sternberg cells in classical Hodgkin lymphoma. **B**. "Popcorn cell" in nodular lymphocyte predominant lymphoma (Haematoxilin and Eosin, original magnification x400)

The cellular origin of the tumor cells in HL remained elusive for many years, since

The cell of origin of HL tumor cells was elusive for many years until analysis of immunoglobulin gene rearrangement in microdissected tumor cells revealed that these cells are

frequently clonal B cells with somatically mutated immunoglobulin V genes (*Kuppers et al, 1994; Kanzler et al, 1996; Kuppers et al, 1994*). However, except of LP cells, that consistently express the B-cell markers CD20, CD79a, CD19, and the transcriptors factors PAX5, OCT2 and BOB1, the phenotype of the H/RS does not reveal its B cell origin. Indeed, apart from low expression of PAX5 and a focal staining for CD20 in about 20% of the cases, all the other markers of B cell lineage are down regulated in cHL (*Tzankov et al, 2003; Saez et al, 2002; Kuppers 2009 Hematol*; *Browne et al, 2006*). Characteristically, H/RS cells and not LP cells express CD30 a member of the tumor necrosis factor receptor (TNFR) family. Also, in about 85% of the cases there is coexpression of CD15, a myeloid-associated marker. Aberrant expression of T-cell (*Dallenbach et al, 1989*; *al Saati et al, 1997; Tzankov et al, 2005*) or dendritic cell-associated markers (*Pinkus et al, 1997; Sorg et al, 1997; Peh et al, 2001*) has also been reported in a minority of cHL. Although most of the cases expressing a T-cell phenotype are also found to be of B-cell origin based on molecular analysis, a T-cell origin has also been suggested in few reported cases based on the presence of T cell receptor gene rearrangements in tumor cells (*Muchen et al 2000; Seitz et al, 2000*).

1.3.2 Epidemiology

Hodgkin lymphoma (HL) is one of the most common lymphomas in the Western Word with an incidence of 3 cases per 100 000 persons-year (*Farrell et al, 2011*). Classical Hodgkin lymphoma accounts for more than 95% of all HL and is associated with EBV in about 30-40% of the cases (*Swerdlow et al, 2008*). The highest incidence of cHL is in Caucasians, followed by African Americans and Hispanics, with the lowest observed in Orientals. However, an increasing incidence of cHL was reported among Chinese immigrants in Western Countries (*Huang et al,*

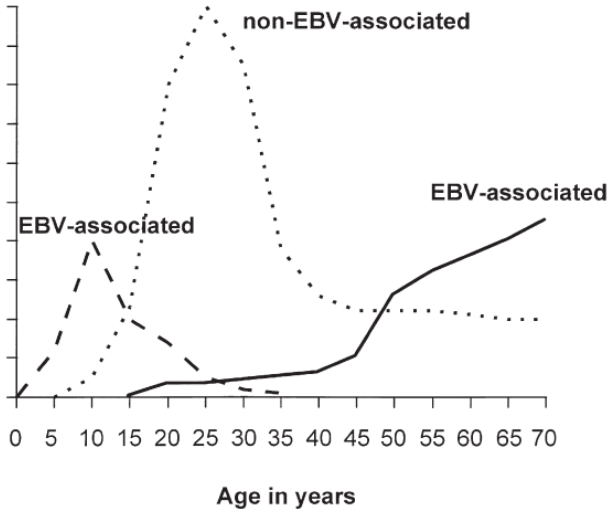

Figure 3 Three-disease model for Hodgkin Lymphoma. *(Armstrong et al. Leukemia (1998)12, 1272-1276)*

2011). Epidemiologic and molecular findings suggest that cHL is not a single disease but consists of more than one entity. Indeed, cHL may occur in different geographic and socioeconomic settings with distinct pathological and clinical characteristics (*Carbone et al, 2011*). In 1998 Armstrong *et al*. proposed a "three-disease model" based on age presentation, histological type and EBV association (Figure 3) (*Armstrong et al, 1998*). The first entity is largely an EBV-associated disease of childhood, with higher incidence in developing countries and of usually MC type. The second entity predominantly affects older adults, and is also usually of the MC subtype and EBV associated. The third one occurs in young adults, is more prevalent in developed countries, is not associated with EBV, and is usually of the NS subtype. Harris *et al*. suggested three epidemiological patterns of cHL based on socioeconomic level. The first pattern is seen in poorly developed countries, occur in early childhood, and is mainly of the MC subtype; the second pattern is observed in developing and transitional economies, affects both children and adolescence, and shows equal frequency of MC and NS subtypes; the third one occurs in developed countries and display a third decade peak and a predominance of NS subtype (*Harris et al, 1998*). Another HL classification is based on patients's immunological status, by which cHL may occur: a) in the general population, or b) in immunosuppressed hosts (associated with HIV-infection or with iatrogenic immunosuppression) (*Carbone et al, 2011*). However, despite all these categorizations, there are several lines of evidence that suggest that these epidemiological patterns are not tight and that transition may exists among different forms of the disease.

1.3.3 Pathogenesis and role of EBV

EBV is found in up to 40% of cHL, whereas is rarely found in NLPHL (*Kuppers et al, Nature 2009*). The association varying with age (more frequent in children and older adults), gender (more frequent in males), geography and socioeconomic status (higher in Asia and in Central and South America), histology (more likely in MC and LD subtypes), and host conditions (nearly all cases of HL occurring in patients with AIDS are EBV+) (*Kuppers et al, Nature 2009; De Re et al, 1993*). In EBV+ tumors EBV is detected in the majority of H/RS cells and it is found to be clonal, suggesting that infection occurred prior to transformation of B cells (*Weiss et al, 1987; Weiss et al, 1989; Gulley et al, 1994*).

EBV+ H/RS cells exhibit a type II latency phenotype, with the expression of a limited number of latency genes, including EBNA1, LMP1, LMP2 and EBERs. EBNA 1 is essential

for maintaining viral genome as an episome. Moreover, it may support tumor development upregulating CCL20, an attracting chemokine for Tregs, and downregulating the protein-tyrosine phosphatase-k, which is a putative tumor suppressor gene (*Baumforth et al, 2008; Flavell et al, 2008*). LMP-1 is an oncogene that mimiking an active CD40 receptor constitutively activates NF-kB in tumor cells (*Kilger et al, 1998*). LMP-2 can replace the function of a BCR, and *in vitro* is essential for the rescue and transformation by EBV of germinal center (GC) B cells lacking functional BCRs (*Bechter et al, 2005*). Thus, it appears that through the BCR and the CD40 signaling EBV may play a critical role in the initial events of HL pathogenesis, rescuing from apoptosis EBV-infected GC cells carrying BCR destructive mutations (*Mancao et al, 2005; Chaganti et al, 2005*). In addition, LMP-1 and LMP-2 contribute to the downregulation of the B-cell phenotype in EBV+ H/RS cells through activation of the Notch1 pathway (*Portis et al, 2003*; *Vockerodt et al, 2008*).

The demonstration that a virus strain carrying a 30bp deletion in the LMP1 gene was more tumourigenic than the prototype B95.8 LMP1 led to numerous studies of the prevalence of this virus strain within EBV-associated cancers, including HL. In general, virus strains carrying this 30bp deletion occur with a similar frequency in virus-positive tumour patients and in healthy donors from the same geographical region. The exception to this is HL, where some studies have shown an increased incidence of this deletion variant in HIV-positive HL compared to HIV-negative HL, and in pediatric HL compared to normal controls (*Hu et al, 1993; Santon et al, 1998*).

Nevertheless, most adults that carry EBV never develop cHL, and in up to 60% of cHL there is no evidence of EBV in malignant cells. Although the morphology, phenotype and gene expression profile of EBV-associated and EBV-negative cases of cHL appear similar, there is increasing evidence that the molecular pathogenesis of these two conditions may be distinct (*Farrell et al 2011*). Based on epidemiological observations, in 1996 Mac Mahon has suggested that Hodgkin's disease may not be a single entity but a syndrome comprising at least two, and possibly three, entities with different etiologies (*MacMahon, 1996*). This hypothesis was further supported by Armstrong *et al* with the "three-disease model" with HL occurring in childhood (EBV+, MC type), HL of young adults (EBV-, NS type) and HL of older adults (EBV+, MC type) (*Armstrong et al, 1998*). In 2002 based on studies assessing that infectious mononucleosis is associated with higher risk of developing HL, Jarrett *et al*. has extended the "three-disease model" to include a fourth entity represented by EBV-associated cases occurring in young adults with delayed exposure to EBV (*Jarrett et al, 2003; Jarrett et al, Leuk Lymphoma 2003*). However, the infrequent association of EBV with HL in young adulthood,

suggested that EBV is mainly not pathogenetic in young patients and that another currently unknown virus might be involved in the development of the disease (*Kapatai et al 2007*, *Jarrett et al, Leuk Lymphoma 2003*). Conversely, the "hit and run hypothesis" (*Ambinder, 2000; Jox et al, 1997; Trivedi et al, 1995*) suggested that EBV may contribute early on the pathogenesis of EBV-negative HL. Based on this hypothesis after EBV has infected and transformed B cells it is lost because of the presence of a defective integrated and rearranged viral DNA. The partial elimination of defective EBV episomes from infected cells has been previously detected in some cases of EBV-negative sporadic Burkitt lymphoma (*Trivedi et al, 1995*); this prompted to the search for EBV DNA in H/RS cells. Although Gan *et al*. found defective EBV genome in 2 cases of EBV-negative HL, others did not found any trace of EBV in H/RS to support a "hit-and-run" mechanism. Moreover, because some young patients with HL have no evidence of previous EBV-infection, it seems unlikely that EBV can be responsible for EBV-negative HL. In this view, EBV-positive and EBV-negative HL may simply represent two distinct diseases (*Gan et al, 2002*; *Gallagher et al, 2003*). Whether or not EBV-positivity in HL has prognostic significance remains controversial, with data on both sides of the question (Table 4) (*Jarrett et al, 2005*); nevertheless, the expression of viral antigens on tumor cells may still be crucial posing theoretical targets for anti-cancer therapies, including vaccination.

Reference	Population-based	No. of Patients	Age range, y	Median follow-up, mo	NLPHL included	% EBV-associated	Prognostic significance of EBV-association on OS
Armstrong et al[5]	No	59	Not stated	Not stated	Yes	36	None
Axdorph et al[6]	No	95	14–77	145	No	33	None
Enblad et al[11]	Yes	117	11–87	130	Yes	27	None
Flavell et al[8]	No	273	≥15	60	Yes	29	None
Murray et al[7]	No	190	22–49	86	Yes	27	None
Vestlev et al[4]	No	66	9–78	22	Yes	41	None
Krugmann et al[9]	No	119	14–83	122	No	26	None
Herling et al[10]	No	303	Adults	65	No	21	None
Vassalo et al[15]	No	78	15–75	Not stated	No	64	Favorable (assessed by LMP1 expression)
Glavina-Durdov et al[16]	No	100	13–84	60	Yes	26	None. Positive impact on DFS in those ≤30 y
Engel et al[13]	No	47	≤14	Not stated	No	68	Favorable
Morente et al[12]	No	140	5–83	>24	Yes	51	Favorable
Naresh et al[14]	No	110	4–61	57	No	78	Favorable
Stark et al[18]	Yes	102	≥60	62	Yes	34	Unfavorable
Clarke et al[17]	Yes	311	19–79	73	Yes	17	Unfavorable in those ≥ 45 y

NLPHL indicates nodular lymphocyte predominant Hodgkin lymphoma; EBV, Epstein Barr virus; OS, overall survival; LMP1, Epstein-Barr virus latent membrane protein 1; and DFS, disease-free survival.

Table 4. Summary of previous studies that have examined the effect of EBV status on clinical outcome in Hodgkin lymphoma. *(Jarrett RF et al. Blood 2005 106: 2444-2451)*

1.3.4 Genetic alterations and deregulated signalling

H/RS cells carry rearranged and somatically mutated Ig V genes in nearly all cases; however in 25% of the cHL clearly destructive somatic mutations were found that rendered originally functional IgV region non functional (*Kuppers et al, 1994; Kanzler et al, 1996*). Such mutations happen in germinal centre (GC) B cells, that normally rapidly undergo apoptosis. Thus, in HL pathogenesis critical steps most likely occur in GC to enable H/RS precursors with defective BCR to escape apoptosis. Nevertheless, it can not be rule out that additional transforming events could affect H/RS cells before they enter the GC and even after they have left it. Supporting this multistep process is the evidence that HL tumor cells usually show multiple chromosomal abnormalities and subclonal aberrations, indicating chromosomal instability (*Steidl et al, 2010; Weber-Matthiesen et al, 1995*).

Chromosomal translocations involving the Ig loci were detected in about 20% of cHL. Some of them involve the oncogenes BCL2, BCL6 and MYC, but for most cases partner gene is unknown (*Martin-Subero et al, 2006*). Considering the general silencing of the Ig loci in H/RS cells, it might be possible that these translocations may be relevant just in the early stages of the disease, when H/RS cells still have a B-cell phenotype. Conversely, translocations involving BCL6 are frequently found in LP cells (*Wlodarska et al, 2003*).

A rescue of H/RS from apoptosis is probably a key event in HL pathogenesis hence, activators or inhibitors of apoptosis were extensively studied for genetic aberrations in H/RS cells. The genetic lesions most frequently found in cHL involve members of two signalling pathways: JAK-STAT and NF-kB. In about 20% of HL cases there are genomic gains of JAK2 in H/RS cells, and in 40% of the cases inactivating mutations of SOCS1, a negative regulator of JAK-STAT signalling, can be found (*Joos et al, 2000; Weniger et al, 2006*). Multiple genetic lesions in the NF-kB pathway seems to contribute to its deregulation in H/RS cells. Interesting, among these an inhibitor of NF-kB activity A20, is inactivated in 40% of mainly EBV-negative HL (*Schmitz et al, 2009*). This suggest that A20 inactivation and EBV infection are mutually exclusive transforming events in cHL. Other cell signalling pathways known to be aberrantly activated in HL include the PI3K/AKT pathway and the MAPK/ERK pathway. Both may be critical for H/RS cell survival and proliferation (*Dutton et al, 2005; Zheng et al, 2003*).

1.3.5 Tumor microenviroment

The microenviroment in HL is unique among lymphomas both for the complexity of cell types involved, and for the fact that non-tumor cells represent the majority of the cells,

accounting for 99% of all the cells in the tumor. H/RS cells grow in a typical microenvironment that is composed of several types of cells, including B cells, T cells, plasma cells, eosinophils, mast cells, and fibroblasts (*Swedlow et al, 2008*). This microenvironment is a critical determinant for the initiation and progression of HL. It is likely essential for H/RS cell survival, as indicated from the difficulty to grow H/RS cells in culture or in immunodeficient mice or to find H/RS cells in the peripheral blood (*Kapp et al, 1993; Kapp et al, Blood 1993*). H/RS cells appear to regulate this microenvironment by secretion of cytokines and chemokines (Figure 4) (*Steidl et al 2011*). For example, H/RS cells attract eosinophils by granulocyte-macrophage colony-stimulating factor, IL5, IL9, CCL5, and CCL28, and they attract TH2-type T helper cells and Treg cells by secretion of CCL5, CCL17, and CCL22 (*Skinnider et al, 2002; Aldinucci et al, 2008*; *Fisher et al, 2003*).

CD4+ T cells usually represent the largest population of cells in the lymphoma tissue. A fraction of these cells are CD4+ T helper cells. These cells are in close contact with H/RS cells and may play a pathogenetic role by stimulating the survival and growth of tumor cells. Indeed, although H/RS cells do not express most B cell-associated genes, they retained expression of MHC class II, CD40, CD80 and CD86, key molecules for an interaction of B cells with T helper cells. The interaction between H/RS cells and T cells is histologically typical; in HL biopsies H/RS cells are surrounded by CD40 ligand-expressing CD4+ T cells forming with them “rosettes” structures. The CD40 stimulation mediated by T cells leads to the activation of NF-kB, a survival signal for H/RS cells (*Carbone et al, 1995; Nozawa et al, 1998*).

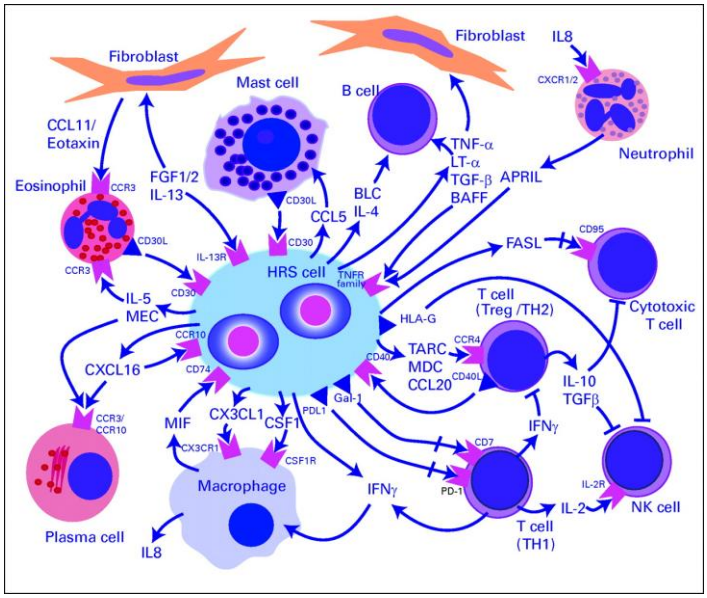

Figure 4. Schematic of the crosstalk between malignant Hodgkin Reed-Sternberg (H/RS) cells and the tumor microenvironment in classical Hodgkin's lymphoma. *(Steidl C et al. JCO 2011;29:1812-1826)*

H/RS cells can also orchestrate the cellular infiltrates to evade an attack by cytotoxic T cells (CTL) or NK cells. In this regard, it has recently become clear that many of the CD4+ T cells in classical HL are not helper but regulatory T cells. H/RS cells not only can attract these cells but also can induce the differentiation of naïve CD4+ T cells into Treg cells (*Tanijiri et al, 2007*). Tregs may have a pathogenetic role, as there is

indication that they have immunosuppessive activity on HL-infiltrating CTL cells (*Marshall et al, 2004*). However, high number of Treg cells in HL microenviroment has been reported to be associated with a better prognosis, indicating that an excess of these cells may have suppressive effects also on H/RS survival (*Alvaro et al, 2005; Kelley et al, 2007*). Furthermore, H/RS cells might inhibit cytotoxic cell functions through the expression of PD1 and CD95, and the secretion of the immunosuppressive cytokines IL-10, TGFβ, galectine-1 and prostaglandin E2 (*Chemintz et al, 2007; Gandhi et al, 2007; Aldinucci et al 2010*; *Kuppers et al 2012*).

EBV infection might also affect the microenviroment composition by increasing the production of molecules involved in immune escape and T cell recruitment. For example, IL10 was found to be expressed in 66% of EBV+ cHL cases but in only 16% of EBV-negative cases (*Skinnider et al 2002*). *In vitro* HL cells can process and present epitopes from LMP1 and LMP2A in the context of multiple class I alleles (MHC) and are sensitive to lysis by EBV-specific CTLs. However, EBV-infected H/RS cells survive *in vivo*, probably because H/RS cells are able to counteract EBV-specific CTL responses. Surprisingly, EBV+ HL have been shown to contain more activated CTLs and express higher levels of MHC class I than EBV-negative cases (*Kapatai et al, 2007*). Moreover, Chetaille *et al*. described a molecular signature of EBV+ cHL, characterized by genes associated with Th1 and antiviral responses (*Chetaille et al, 2009*). However, CTL cells might not be effective in the immune response against EBV in H/RS cells. Recent data have shown a strong association between EBV+ HL and human leukocyte antigen (HLA) class I genotype. Increased risk was associated with HLA-A*01 and decreased risk with HLA-A*02. Whereas CTL responses to many HLA-A*02 restricted EBV epitopes have been described, there are no confirmed HLA-A*01-restricted responses to epitopes derived from either lytic or latent viral proteins. This raises the suspicion that the increased risk of EBV-associated cHL is related to a weak EBV-specific CTL response (*Hjalgrim et al, 2010; Straathof et al, 2005*).

Several studies point to an adverse prognosis for EBV-association in adult cHL; however, physiological and age-related changes of the immune system may also play an important role in modulating the tumour microenvironment in HL. Supporting this, a favourable outcome was described for EBV+ cHL occurring in young patients (*Barros et al, 2012*). Also, another study has demonstrated that there is an age-dependent relationship between tumor EBV status and clinical outcome in cHL. In particular, it was found that in patients aged 16 to 49 years EBV status has not significant impact on prognosis, whereas in patients over 50 years EBV status was significantly associated with poorer outcome, suggesting that an age-related decrease in

immunity may contribute to the age effect on prognosis in patients with EBV+ cHL (*Jarrett et al, 2005*).

2. PERSONAL CONTRIBUTION

2.1AIM

Based on epidemiological studies, EBV-positive lymphoproliferative disorders (LPD) are associated with patient's age, immune system status of the host, geography, and socioeconomic conditions. In particular, in immunocompetent patients EBV+ cHL occur more frequently in children from poorly developed countries and in older adults from developed countries, whereas EBV-negative cases are more frequent among young adults of developed countries (*Armstrong et al 1998, Jarrett et al 2003*). These differences have questioned the effective role of EBV in the pathogenesis of the disease, and have raised the possibility that EBV-positive and EBV-negative cHL may represent two distinct diseases (*Harris et al 1998*).

It has been suggested that different factors may contribute to the development of EBV+ cHL in children and older patients. In the former, early age of EBV infection has been reported to greatly affect the association of EBV with cHL (*Glaser et al 1997*) while, in the latter immunosenescence related to patient's age has been proposed as a key factor for the development of EBV+ cHL (*Jarrett* et al *2005, Dojcinov et al 2011*). In both cases, it has been suggested that an impaired immune status of the host may contribute to the development of EBV+ cHL. However, whether or not EBV+ cHL occurring in children and in old patients represent the same disease remains to be clarified.

To address this issue, we characterized and compared the immunophenotipic and molecular features of 57 cases of HL occurring in pediatric patients from Baghdad with those of 30 cases of HL diagnosed in old Italian patients.

2.2 MATERIALS AND METHODS

Patients

Paraffin blocks of 57 cases of pediatric HL first diagnosed at the Children Welfare Teaching Hospital of Baghdad in the period 2008-2012 were sent to the Pathology Unit of Sant'Andrea Hospital of Rome for a second opinion. Clinico-pathological features of the patients are summarized in Table 5. Histologically, 51 cases were classified as cHL (89%), of which 35 as MC (69%), and 16 as NS (31%). Six cases were classified as NLP-HL. Moreover, 30 cases of Hodgkin Lymphoma diagnosed in caucasian Italian patients aged 50 years or older in the period 2003-2012 at Sant'Andrea Hospital of Rome were enrolled in the study (Table 7). A control group of 10 cases of EBV-negative cHL from caucasian Italian adult young patients (age range 17-32 year old) was included in the study.

Immunohistochemistry

Phenotypic characterization of tumor cells was performed by immunohistochemistry on formalin-fixed paraffin-embedded (FFPE) serial sections of the involved lymph node using the following antibody: CD3, CD4, CD8, CD20, (Novocastra, UK), CD30, CD79a, CD15, (Dako, Denmark), PAX5 (Thermo Scientific, USA), OCT2 and BOB1 (Santa Cruz Biotechnology Inc., USA). The number of stained cells with H/RS morphology was determined at 400X. Cases were classified as negative; or as having <50% H/RS cells stained; or as having >50% H/RS cells stained.

T-cell components present in the tumor microenvironment of cHL was investigated by immunohistochemistry for CD3, CD4, CD8, CD56, and Granzyme B (Dako, Denmark). Data were obtained by counting 5 separate 1000× high-power fields (HPFs) and calculating the mean number of positively stained cells per HPF.

FFPE tissue sections were immunostained on an automated immunostainer (Dako Corp., Carpinteria, CA) using a dextran polymer-peroxidase-DAB detection kit (Dako EnVision™ FLEX+ kit) according to the manufacturer's instructions. Briefly, using a 3-in-1 procedure, deparaffinization, rehydratation and heat-induced epitope retrieval was performed incubating 4 μm thick paraffin sections with the Envision pre-heated target retrieval solution for 20 minutes at 97°C. Sections were then cooled at room temperature, immersed in a washing

buffer for 5 min, and then incubated with an optimal dilution of the primary antibody for 20 min. After a washing bath, sections were incubated for 20 min with a FLEX HRP-conjugated (horse radish peroxidase) linker composed by secondary antibodies with anti-mouse and anti-rabbit Ig specificity. Immunohistochemical reactions were subsequently developed with diaminobenzidine as chromogenic peroxidase substrate, and slides were counterstained with haematoxylin.

Tests for the detection of EBV in tumor cells

To investigate the presence of EBV infection in H/RS cells immunohistochemistry for LMP-1 protein (Dako, Denmark) was performed in all the pediatric and adult cHL. In addition, all the cases were also tested by in situ hybridization (ISH) for EBV-encoded RNAs (EBERs). EBER-ISH was performed on paraffin sections using a cocktail of EBER1 and EBER2 fluorochrome-conugated riboprobes (EBER PNA Probe/Fluorescein, Dako, Denmark), and a polyclonal rabbit anti-FITC/HRP antibody (Dako, Denmark). Briefly, deparaffinized 2 μm thick sections were pre-treated with a pepsin solution (proteinase K) for 6 min at room temperature in a humid chamber. Proteolytic process was stopped with 95% ethanol for 10 secs. Sections were washed in distilled water, and air-dried. Sections were then incubated with the EBER PNA probe fluorescein-conjugated for 2 h at 37°C in a hybridization oven and covered with a coverslip. Slides were washed for 25 min with a stringent washing solution, air-dried, and then incubated in a humidity chamber with an anti-FITC/HRP rabbit polyclonal antibody for 30 min. After a washing step with PBS buffer, a 5 min incubation with the HRP-substrate (diaminobenzidina) was performed. Slides were counterstained with haematoxilyn.

Genomic DNA extraction

Genomic DNA was purified from formalin-fixed paraffin embedded tissues using the QIAmp DNA mini kit (Qiagen). The QIAamp DNA purification procedure was carried out using QIAamp Mini spin columns. For each tumor a paraffin block was cut at 10 μm and collected in an autoclaved plastic microtube (1.5 ml). For each microtube, 2 sections of total 20 μm thickness were carefully collected. Deparaffinization was carried out by adding 1 ml xylene to each microtube for 30 min for two changes, followed by 100% and 75% ethanol for 30 min

with two changes. After a washing step with PBS for 15 min in two changes, 200 µl of lysis buffer containing proteinase K was added and incubated at 56 °C overnight using a heat block. When all tissue fragments were dissolved completely, the lysate was loaded onto the QIAamp Mini spin column. A brief centrifugation was carried on to allow DNA to be adsorbed onto the QIAamp silica membrane. DNA bound to the QIAamp membrane was washed in 2 centrifugations using 2 different washing buffers. Purified DNA was eluted in AE from the QIAamp Mini spin column in a concentrated form. Using a spectrophotometer (Nanodrop, Thermoscientific), the amount of DNA yield was measured according to the standard protocol recommended by the manufacturer.

Polymerase Chain Reaction (PCR) analysis of the LMP-1 gene polymorphism

To investigate LMP-1 gene polymorphism in HL biopsies of Iraqi children, the DNA was extracted from 25 EBV+ cHL (21 MC and 4 NS) as previously described. The DNA from B95.8 and Raji cell lines was used as LMP1 wild-type control. The DNA from AG876 and Rael cell lines was used as LMP1 deleted variant control. A DLBCL cell line, U2932 was used as EBV negative control of the PCR reaction. For the PCR reaction, following primers were designed: forward primer: 5' GTG GGG GTC GTC ATC ATC TC 3' (B95.8 coordinates 168190-168209); reverse primer: 5'CGG AAG AGG TTG AAA ACA AA 3' (B95.8 coordinates 168331-168350). The DNA was amplified using the following PCR conditions: initial denaturation step at 94°C for 5 minutes, 40 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 1 minute, final extension step at 72°C for 10 minutes. PCR products were visualised on a 2% agarose gel. The product length for the wild type LMP1 amplicon was 161 bp, and for the deleted LMP1 variant amplicon 131 bp.

PCR detection of Epstein Barr virus (EBV)-encoded RNA 1 (EBER1)

In order to further confirm the presence of EBV-infection in HL biopsies of two elderly patients with methachronous B-cell lymphoma a PCR for the detection of EBER1 gene was performed on total DNA extracted from the whole paraffin tissue sections. For the PCR reaction, the following primers were designed: forward primer 5'AGGACCTACGTGCCCTAGA3', reverse primer 5'AAAACATGCGGACCACCAGC3'.

The DNA from U2932 EBVGFP cl1 (DLBCL, EBV+) and RAJI (BL, EBV+) cell lines was used as EBER1-positive control. U2932 (DLBCL, EBV-) and BC3 (PEL, EBV-) cell lines were used as EBV-negative controls of the PCR reaction. PCR products were visualised on a 2% agarose gel. The product length for EBER1 amplicon was 167 bp.

T-cell receptor-gamma (TCR-γ) gene rearrangements analysis

In order to investigate the status of the TCRγ gene in both the pediatric and the elderly cases of HL, a TCRγ gene clonality assay was carried out using the "TCRγ rearrangements molecular analysis kit" (Master Diagnostica, Spain) in 38 of 51 Iraqi cHL and in 21 of 30 cases of adult cHL. This test utilizes the BIOMED-2 multiplex PCR master mixes targeting the variable (V) and joining (J) regions that flank the unique hypervariable antigen-binding region 3 (CDR3) of the TCRγ gene locus, which is rearranged early during T-cell development (*van Dongen et al 2003*). In particular, genomic DNA extracted from paraffin tissue sections was used in two independent PCR amplification reactions. One reaction amplified DNA sequences between the V gamma segments 1-8 and 10, and all J gamma segments, while the other amplified DNA sequences between the V gamma segments 9 and 11, and all J gamma segments. One monoclonal and one polyclonal control samples were included in each reaction Following PCR amplification, the fluorochrome-labeled single-strand (denatured) PCR products were separated in a capillary sequencing polymer as function of size and detected automatically with a laser scanning on the ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, California). This analysis is based on the principle that primers conjugated with different fluorescent dyes produce different emission spectra upon excitation by a laser in a capillary electrophoresis instrument (GENESCAN). In this manner, different fluorophors can correspond to different targeted regions. GeneMapper software (ABI) was then used to visualize and analyze PCR products, allowing detection of clonal cells. In case of polyclonal lymphoproliferation many different PCR products of different sizes resulted in a gaussian distribution of homogeneous peaks. One type of PCR product due to a fully monoclonal lymphoid population gave a single prominent peak. When two unequivocal peaks were observed the case was considered as bi-allelic monoclonal. Samples in which more than 2 discrete peaks (2.5-fold higher than the adjacent peaks that represent the polyclonal background) were observed were scored as oligoclonal (Figure 6).The limit of detection of this assay has been determined by the manufacturer to be approximately 1 clonal cell in 100

hundred normal cells (1%), and inter-assay and intra-assay reproducibility in size determination using capillary electrophoresis has been considered to be approximately 1-2 basepairs.

Heteroduplex analysis of TCRγ **gene** *rearrangements*

PCR amplifications products of TCRγ gene were also evaluated by heteroduplex analysis on a nondenaturing polyacrylamide gel according to BIOMED-2 report (*van Dongen et al 2003*). Twenty µl of PCR product was denaturated at 94ºC for 5 minutes and re-annealed at 4ºC for 60 minutes. Five µl of ice-cold non-denaturing bromophenol blue loading buffer was added to samples. Then 20µl of mixture was loaded into the wells of 6% non-denaturing polyacrylamide TBE gel (Invitrogen) with a 0.5X TBE running buffer (Invitrogen) and runned at 110V for 3 hours. Gels were stained in 0.5µg/ml EtBr in water for 5-10 minutes, and washed twice in water for 5-10 minutes. UV illumination was used for visualization. Gel was photographed and data were interpreted. In heteroduplex analysis, PCR products are heat denatured and subsequently rapidly cooled to induce duplex (homo or heteroduplex) formation. In samples that contain polyclonal lymphoid cells PCR fragments of rearranged TCRγ genes form heteroduplexes, which result in a background smear of slow migrating fragments. PCR products from monoclonal or oligoclonal lymphoid cell populations give rise to homoduplexes bands (Figure 6).

Immunoglobulin (IGH) gene rearrangements analysis

We performed molecular evaluation of IGH gene rearrangements in 38 of 51 Iraqi cHL and in 21 of 30 cases of adult cHL using the "Identyclone IGH gene clonality assay" (Invivoscribe, California). This test, based on BIOMED 2 protocol (Ref 15), amplify the DNA between primers that target the three conserved framework regions (FR1, FR2, and FR3) of the variable (V) segments and the conserved joining (J) regions of the IGH gene locus. These regions rearrange during B-cell differentiation generating VDJ products of unique length and sequence. Genomic DNA extracted from FFPE tissues was amplified using three master mixes that combine 3 different sets of VH primers with 1 JH consensus primer. PCR amplifications products were then analyzed on the ABI 3130 Genetic Analyzer (Applied Biosystems, Foster

City, California) using GeneMapper program (as previously described for the TCRγ gene clonality assay). One monoclonal and one polyclonal control samples were included in each reaction. Clonal rearrangements were identified as prominent, single-sized peaks in the expected range, whereas samples were scored as polyclonal when a gaussian distribuition of homogeneous peaks was observed.

Laser Capture Microdissection and TCRγ gene rearrangement analysis

Laser capture microdissection was performed using the microdissection laser system SL CUT (NIKON Instruments, Italy). Large atypical cells with Hodgkin and Reed-Stenberg-like morphology were isolated on Haematoxylin and Eosin stained sections collecting approximately 200 cells per sample. Moreover, about 300 morphologically typical lymphocytes were picked from the same tissue section. DNA extraction was performed using the Pico pure isolation Kit (ARCTURUS, Bioscience Inc., Mountain View, CA, USA). TCRγ clonality assay was performed on microdissected samples as previously described for the whole tissue sections.

Statistical analysis

The association between clinical, pathological, and molecular variables (tumor histology, EBV-infection, TCRγ and IGH clonality) was assessed using Fisher's exact test. Student's t-test was used to test the correlation between patient's age and tumor histology. Associations were considered to be statistically significant with p values < 0.05.

2.3 RESULTS

Clinico-pathological features of HL occurring in Iraqi children

In a collaborative study between the Children Welfare Teaching Hospital of the University of Baghdad and the Sapienza University of Rome, 57 cases of Hodgkin lymphoma (HL) affecting Iraqi children under 14 years of age were reviewed at the Sant'Andrea Hospital of Rome (Table 5). Histologically, 51 cases were classified as cHL (MC = 69%; NS = 31%), and 6 cases as Nodular Lymphocyte Predominance HL. The children with MC were predominantly male (ratio M:F MC=6:1 versus NS=1.3:1; p=0.033), and were younger than those with NS (mean age = 6.9 year old versus 8.75 year old; p=0.016).

Table 5. Age-related histology and EBV infection of 51 cases of pediatric cHL.

Groups	M:F Ratio	n. of cases	MC-HL	NS-HL	EBV+
3-5y	5:1	18	16 (89%)	2 (11%)	18 (100%)
6-10y	3:1	24	14 (58%)	10 (42%)	20 (83%)
11-13y	2:1	9	5 (56%)	4 (44%)	6 (67%)
Total	3.25:1	51	35 (69%)	16 (31%)	44 (86%)

To detect EBV infection of H/RS cells EBER hybridization and LMP-1 immunostaining was performed in all the cases (Fig. 5a). EBV infection of H/RS cells was demonstrated in 44 of 51 cases of cHL (86%), and was more common in MC than in NS (97% versus 63%; p=0.0025); the 6 cases of NLP-HL were all EBV-negative. In all EBV+ cases, LMP-1/EBER reactivity was detected in CD30-positive cells with typical H/RS morphology (Figure 5); EBV+ H/RS cells were 60% of CD30+ cells in MC and 53% in NS. When children were stratified according to age it was found that all cases of cHL in the 3-5 years age range were EBV+. Moreover, with the increase of the age, there was a gradual decrease of EBV+ cases (from 100% to 67%), and an increase in NS cases (from 11% to 44%). These date indicate a progressive age-related switch from an EBV+ MC type of cHL to an EBV-negative NS type.

Immunohistochemistry for CD20, CD79a, PAX-5, OCT-2, and BOB-1 was used to assess the immunophenotypic profile of H/RS cells. Expression of B cell markers by H/RS cells was similar in MC and NS, and was not influenced by EBV infection. In fact, H/RS cells were

PAX-5 positive in the majority of cHL cases (MC 94% versus NS 87%), and expressed at a lesser degree other B-cell markers including CD20 (MC 26% versus NS 25%), CD79a (MC 37% versus NS 25%), OCT-2 (MC 31% versus NS 12%), and BOB-1 (MC 11% versus NS 12%) (Table 6). The six cases classified as NLP-HL were all EBV-negative, and contained LP cells intensely positive for all the B–cell markers and negative for CD30 and CD15 (Table 6).

Table 6. Expression of B cell markers by H/RS cells in 57 cases of HL from Iraqi children.

Histology	n. of cases	PAX5			CD20			CD79a			OCT-2			BOB-1		
		Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%
cHL-MC	35	2 6%	10 28%	23 66%	26 74%	9 26%	0 -	22 63%	13 37%	0 -	24 69%	8 23%	3 8%	31 89%	4 11%	0 -
cHL-NS	16	2 13%	3 19%	11 68%	12 75%	4 25%	0 -	12 75%	3 19%	1 6%	14 88%	1 6%	1 6%	14 88%	2 12%	0 -
cHL-EBV-pos	44	4 9%	11 25%	29 66%	33 75%	11 25%	0 -	27 61%	16 36%	1 2%	32 73%	8 18%	4 9%	39 89%	5 11%	0 -
cHL-EBV-neg	7	0 -	2 29%	5 71%	5 71%	2 29%	0 -	7 100%	0 -	0 -	6 86%	1 14%	0 0%	6 86%	1 14%	0 -
NLP-HL	6	0 -	1 17%	5 83%	0 -	0 -	6 100%	0 -	0 -	6 100%	0 -	0 -	6 100%	0 -	0 -	6 100%

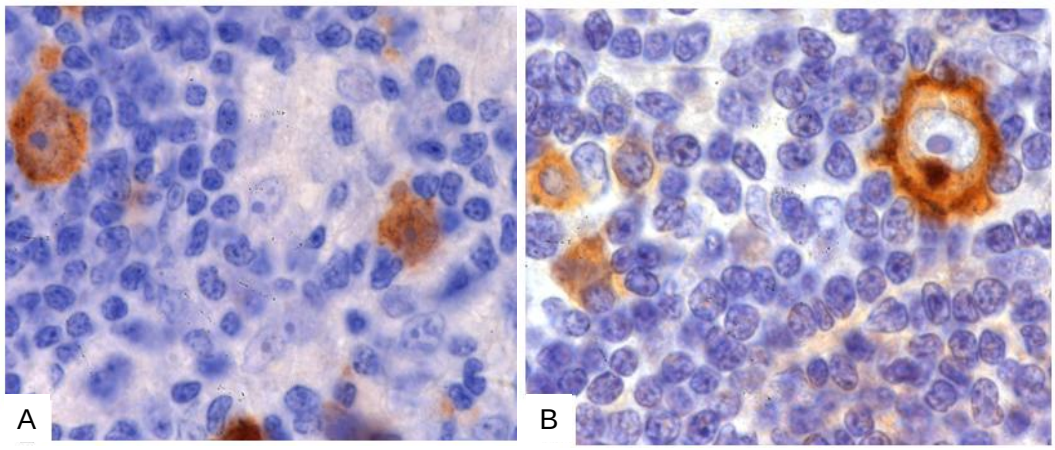

Figure 5. Paraffin section of a lymph node involved by a pediatric MC cHL immunostained for LMP-1 (A), and CD30 (B) (original magnification x400).

Table 7. Histology and EBV infection in 30 Italian cHL stratified according to age

Groups	M:F ratio	n° of cases	MC-HL	NS-HL	LR-HL	Uncl-HL	EBV+
50-59y	1:1	8	2 (25%)	3 (38%)	2 (25%)	1 (13%)	3 (38%)
60-69y	1:1	10	2(20%)	6 (60%)	2 (20%)	0	4 (40%)
70-79y	7:1	8	4 (50.0%)	2 (25.0%)	1 (12.5%)	1 (12.5%)	5 (62.5%)
≥80y	0:4	4	3 (75%)	1 (25%)	0	0	4 (100%)
Total	-	30	11 (37%)	12 (40%)	5 (17%)	2 (7%)	16 (53%)

Clinico-pathological features of HL occurring in Italian old adults

Thirty cases of classical Hodgkin lymphoma diagnosed in patients over 50 years of age at Sant'Andrea Hospital of Rome were further characterized. Histologically, they were subclassified as MC in 11 cases (37%), as NS in 12 cases (40%), and as LR in 5 cases (17%). In 2 cases biopsy samples were too small to assess HL variant and were identified as Unclassified-HL. Compared to NS, MC cases were slightly more common in older patients (mean age MC = 71.6 year old versus NS = 64.3 year old; p=0.057), and were not significantly associated with patients gender (ratio M:F MC=1.3:1 versus NS=1:1; p=1).

EBV infection of H/RS cells was found in 16 of 30 cHL (53%), and was more common in MC than in NS (82% versus 33%; p=0.036). Among the EBV+ cases there were also the 2 Unclassified HL (100%) and 1 case of LR (20%). LMP-1/EBER reactivity was detected in cells with typical H/RS morphology in all the 16 EBV+ cases. EBV+ H/RS cells were 100% of the CD30+ cells in MC cases and 75% in NS cases. When patients were stratified according to age (Table 7), it was found that with increasing age there was a progressive transition from EBV-negative to EBV-positive cases (from 38% to 100%), and an increase in MC cases (from 25% to 75%). This result was exactly the opposite of what we observed in Iraqi children, where it was found an inverse age-related transition from EBV+ MC cases to EBV-negative NS cases.

Concordantly with the literature (*Swerdlow et al 2008; Mani et al 2009*) expression of B-cell markers was found to be downregulated in H/RS cells. In fact, tumor cells were

consistently CD30 and CD15 positive (100% of the cases), expressed frequently PAX-5 (MC 100% versus NS 92%), and at a lesser degree other B-cell markers including CD20 (MC 36% versus NS 8%), CD79a (MC 27% versus NS 25%), OCT-2 (MC 73% versus NS 50%), and BOB-1 (MC 54% versus NS 16) (Table 8). As for pediatric cHL, it was found that the immunophenotypic profile of H/RS cells was similar in MC and NS, and was not influenced by EBV infection. When results where compared to that of Iraqi children, a significantly higher expression of OCT-2 and BOB-1 was found in MC cHL occurring in elderly Italian patients than that found in MC pediatric cases (Table 9).

Table 8. Expression of B cell markers in 30 cHL of Italian old adult patients

Histology	n. of cases	PAX5			CD20			CD79a			OCT-2			BOB-1		
		Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%
cHL-MC	11	0	1	10	7	3	1	8	1	2	3	3	5	5	4	2
		-	9%	91%	64%	27%	9%	73%	9%	18%	27%	27%	45%	45%	36%	18%
cHL-NS	12	1	3	8	11	0	1	9	3	0	6	3	3	10	1	1
		8%	25%	67%	92%	-	8%	75%	25%	-	50%	25%	25%	84	8%	8%
cHL-LR	5	1	1	3	2	2	1	2	2	1	2	2	1	4	1	0
		20%	20%	60%	40%	40%	20%	40%	40%	20%	40%	40%	20%	80%	20%	-
cHL-Unclass	2	0	1	1	0	1	1	1	0	1	1	0	1	1	0	1
		-	50%	50%	-	50%	50%	50%	-	50%	50%	-	50%	50%	-	50%
cHL-EBV+	16	0	3	13	8	4	4	9	3	4	4	4	8	10	4	2
		-	19%	81%	50%	25%	25%	56%	19%	25%	25%	25%	50%	63%	25%	13%
cHL-EBV-neg	14	2	3	9	12	2	0	11	3	0	8	4	2	10	2	2
		14%	21%	64%	86%	14%	-	79%	21%	-	57%	29%	14%	71%	14%	14%

Tumor microenviroment composition in pediatric and elderly cHL

Recently Barros *et al* have shown that in EBV+ pediatric cHL the tumor microenviroment is characterized by a cytotoxic/T-helper cell 1 (Th1) profile (*Barros et al, IJC 2011)*. The different T cell components present in the tumor microenvironment of our series of cHL were investigated by immunohistochemistry for CD4, CD8, CD56, and Granzyme B to identify activated cytotoxic T/NK lymphocytes (CTLs).

In the pediatric cases increased GrB+ CTLs were significantly associated with patient's age of 3–5 years as compared to over 6 years (mean value 129/HPF *vs* 27/HPF, p=0.002), with the histological subtype MC as compared to NS (mean value 104/HPF *vs* 26/HPF, p =0.02) and EBV+ versus EBV-negative status (mean value 95/HPF *vs* 21/HPF, p =0.04); no statistically

significant correlation was found between the number of intratumoral GrB+ CTLs and TCRγ clonality. Consistently with data obtained in the pediatric cases, in cHL of the elderly we found a significant increase of GrB+ CTLs in the MC as compared to the NS subtype (mean value 125/HPF vs. 23/HPF, p =0.005). Conversely, in cHL of the elderly the number of GrB+ CTLs was not associated with EBV-status, age of the patient, and TCRγ gene clonality. No statistically significant differences were found between cHL of Iraqi children and that of old Italian patients (mean value 73/HPF *vs* 66/HPF; p=0.425).

The number of CD4+, CD8+ and of CD56+ cells was comparable between pediatric and elderly cHL. Furthermore, for both pediatric and old patients no association was found between the number of the CD4+, CD8+, and CD56+ cells and histological variant, EBV status and TCRγ gene rearrangements.

Table 9. Expression of B cell markers in MC and NS cHL occurring in Iraqi children and in Italian old patients

Histology	n. of cases	PAX5			CD20			CD79a			OCT-2			BOB-1		
		Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%
cHL-MC old	11	0	1	10	7	3	1	8	1	2	3	3	5	5	4	2
		-	9%	91%	64%	27%	9%	73%	9%	18%	27%	27%	45%	45%	36%	18%
cHL-MC children	35	2	10	23	26	9	0	22	13	0	24	8	3	31	4	0
		6%	29%	66%	74%	26%	-	63%	37%	-	69%	23%	9%	89%	11%	-
		p=0.343			p=0.338			p=0.024			p=0.009			p=0.003		
cHL-NS old	12	1	3	8	11	0	1	9	3	0	6	3	3	10	1	1
		8%	25%	67%	92%	-	8%	75%	25%	-	50%	25%	25%	84	8%	8%
cHL-NS children	16	2	3	11	12	4	0	12	3	1	14	1	1	14	2	0
		13%	19%	69%	75%	25%	-	75%	19%	6%	88%	6%	6%	88%	13%	-
		p=0.999			p=0.06			p=0.999			p=0.072			p=0.560		

LMP-1 gene polymorphism in EBV+ cHL of Iraqi children

Sequence analyses have found several nucleotide variations in the EBV LMP1 gene which has been reported to have greater tumorigenic potential (*Santon et al 1998*). We have investigated the EBV-LMP-1 gene polymorphism in 25 cases of cHL of Iraqi children to demonstrate the characteristic 30 basepair deletion in the 3' end of EBV LMP-1 gene. Wild type LMP-1 gene was found in 17/25 cases (68%), whereas the LMP-1 deleted variant of EBV was detected in 8/25 cases (32%); no significant association was found between the presence of LMP-1 deletion and any of the clinico-pathological features of the disease. Furthermore, the

observed incidence of EBV LMP-1 gene deletion (32%) is similar to that previously reported for USA and Brazil (33%) (*Hayashi et al 1997*).

Table 10. IGH and TCRγ gene rearrangements in cHL of Iraqi childhood

Groups	n. of cases	IGH		TCRγ			IGH + TCRγ
		Monoclonal	Polyclonal	Monoclonal	Oligoclonal	Polyclonal	Monoclonal
cHL all cases	38	14 37%	24 63%	10 26%	18 47%	10 26%	4 10%
cHL-MC	26	10 38%	16 62%	4 15%	14 54%	8 31%	2 8%
cHL-NS	12	4 33%	8 67%	6 50%	4 33%	2 17%	2 20%
cHL-EBV+	33	13 40%	20 60%	6 18%	17 52%	10 30%	3 9%
cHL-EBV-	5	1 20%	4 80%	4 80%	1 20%	0 -	1 20%

IGH and TCRγ gene rearrangements in elderly and pediatric cHL

IGH and TCRγ gene rearrangements were investigated in 38 of 51 cases of pediatric cHL (Table 10) and in 21 of 30 cases of adult cHL (Table 11) using a multiplex PCR on DNA extracted from whole paraffin sections. In the remaining cases (13 pediatric cHL and 9 cHL of the elderly) the analysis was inconclusive, probably because of poor quality DNA. PCR amplifications products of TCRγ gene were also evaluated by heteroduplex analysis on a polyacrilamide gel to confirm the existence of oligoclonal and monoclonal gene rearrangements (Figure 6).

Among the 38 cHL of childhood clonal IGH rearrangements were detected in 14 cases (37%), and oligoclonal/monoclonal TCRγ rearrangements in 28 cases (74%), including 5 of 5 EBV-negative cases. Dual IGH and TCRγ clonal rearrangements were detected in 4 of 38 cases (10%).

In the Italian elderly cases IGH monoclonal rearrangements were found in 10 of 21 cHL (48%). Mono/oligoclonal TCRγ rearrangements were detected in 7 of 21 cases (33%); this pattern was significantly different from what observed in cHL of Iraqi children (p=0.005), where a restricted TCRγ was found in the majority of the cases (74%). In 3 elderly cHLs (14%) a concomitant IGH and TCRγ monoclonal rearrangement was detected.

In both, cHL of childhood and cHL of the elderly, no association was found between IGH and/or TCRγ clonality and histological subtype, EBV status, or patient's age (Table 12). Nevertheless, it is interesting to note that the pattern of TCRγ clonality identified in our series of pediatric and elderly cHL was profoundly different from what we observed in 10 cases of EBV-negative cHL-NS occurred in Italian young adult patients, which exhibited polyclonal TCRγ rearrangements in all the cases (Table 12).

Table 11. IGH and TCRγ gene rearrangements in elderly Italian cHL

Groups	n. of cases	IGH		TCRγ			IGH + TCRγ
		Monoclonal	Polyclonal	Monoclonal	Oligoclonal	Polyclonal	Monoclonal
cHL all cases	21	10 48%	11 52%	5 24%	2 9%	14 67%	3 14%
cHL-MC	8	3 38%	5 62%	2 25%	1 13%	5 62%	1 13%
cHL-NS	8	4 50%	4 50%	2 50%	1 33%	5 17%	1 13%
cHL-LR	3	1 33%	2 67%	0 -	0 -	 100%	0 -
cHL-UNCL	2	2 100%	0 -	1 50%	0 -	1 50%	1 50%
cHL-EBV+	10	5 50%	5 50%	3 30%	1 10%	6 60%	2 20%
cHL-EBV-	11	5 45%	6 55%	2 18%	1 9%	8 73%	1 9%

Immunophenotipic characterization and single-cell PCR analysis of cHL with TCRγ monoclonality

TCRγ gene rearrangements are likely to be related to the non-neoplastic T-cell population that characterized Hodgkin lymphomas however, rarely, T-cell markers-positive H/RS cells showed rearranged TCR genes, suggesting a T-cell derivation in a minority of the cases (*Seitz et al 2000*). To clarify the phenotype of H/RS cells in our series of pediatric and elderly HL with evidence of TCRγ gene monoclonality we investigated the immunohistochemical expression of several B-cell (CD20, CD79a, OCT2, BOB1) and T-cell markers (CD3, CD4, CD4) on H/RS cells. In 2 out of 10 pediatric cases (20%) and in 5 of 5

elderly patients (100%) H/RS showed expression of at least one of the B-cell marker tested; in contrast, none of the cases showed expression of T-cell associated markers on H/RS cells.

To investigate the possibility that TCRγ monoclonality may also be a specific characteristic of H/RS cells, a single-cell PCR analysis of TCRγ gene clonality was performed in 4 EBV+ MC-HL of Iraqi children and in 3 elderly EBV+ MC-HL with evidence of restricted TCRγ gene rearrangements and no expression of T-cell markers on tumor cells. H/RS cells were easily recognizable by morphology, and could be singly collected with laser capture microdissection. DNA was obtained from about 200 microdissected H/RS cells and from 300 microdissected surrounding small lymphocytes per sample. H/RS-like cells and neoplastic T-lymphocytes microdissected from an angioimmunoblastic T-cell lymphoma were used as controls. Interpretable results were yielded only in one case of Iraqi children because of poor quality of the DNA extracted. Both genescan and heteroduplex analyses revealed the presence of a clonal TCRγ rearrangement in the microdissected H/RS cells undistinguishable from that observed in the whole tissue section (Figure 7A). This result suggests that H/RS cells might be able to rearrange both IGH and TCRγ genes. This observation is in keeping with what we have found in a previous study of a case of EBV+ mucocutaneus ulcer (*Di Napoli et al, 2011*). The mucocutaneus ulcer is a newly described EBV-related lymphoma (*Dojcnov et al, 2010*) that affects immunosuppressed or elderly people. It consists of an isolated ulcer, involving the skin or the mucosa of the gastrointestinal tract, composed by a polymorphous inflammatory-like infiltrate, in which are admixed atypical large EBV+ B cells with H/RS-like morphology. IGH and TCRγ genes clonality has been reported in 39% and 38% of the case respectively. We provided evidence of monoclonal TCRγ rearrangement in the microdissected neoplastic cells with H/RS-like morphology and not in the sourrounding lymphocytes (Figure 7B).

Methachronous EBV-positive HL and EBV-negative non-Hodgkin lymphomas

In our series of cHL occurred in elderly patients we found that two patients had a prior history of lymphoma diagnosed by our Institution. The first patient had an extranodal diffuse large B cell lymphoma (DLBCL) of the lung treated with R-CHOP 5 years earlier; the other patient had a small B-cell lymphoma of the MALT type of the left kidney treated with surgery alone 2 years earlier. Both patients had a nodal EBV+ cHL; however, when we look for EBV-infection in the prior extranodal non-Hodgkin B-cell lymphomas we did not find any EBER and/or LMP1 positive cell. To confirm the data we performed PCR analysis for the detection of

EBER-1 gene on the DNA extracted from the tissue biopsies of the methachronous lymphomas. In both patients molecular analysis confirmed the absence of EBER-1 in the previous non-Hodgkin B-cell lymphomas (Figure 8).

IGH gene clonality assay showed monoclonal rearrangements in both patients with EBV+ cHL. In order to establish a possible clonal relationship with previous non-Hodgkin B cell lymphomas we compared rearrangements patterns between the methacronous tumors. In the MALT B cell lymphoma pherograms showed an identical pattern of IGH clonality between the methachronous tumors (Figure 9 case B); whereas, in the large B cell lymphoma the position of the IGH clonal peak differ from the one detected in the subsequent HL for 3 base pairs (Figure 9 case A).

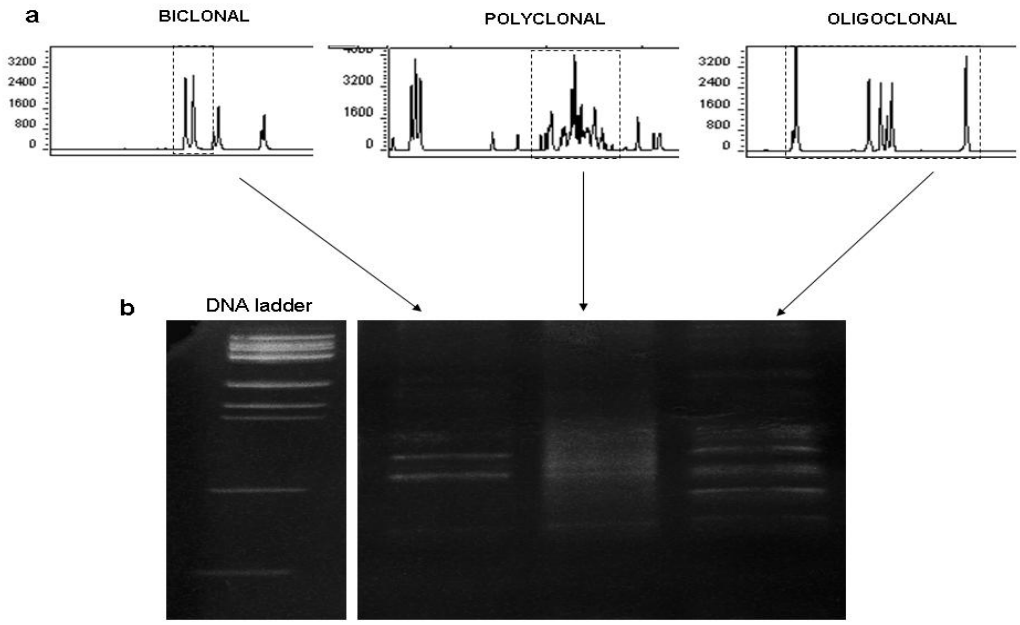

Figure 6. TCRγ gene clonality by Gene Scan fragment analysis (a) and, by heteroduplex analysis (b) of Iraqi pediatric cHL. (a) Samples were scored as monoclonal when one or two (bi-clonal) unequivocal peaks/bands were observed; as polyclonal when a gaussian distribuition of omogeneous peaks/bands was observed and, as oligoclonal when more than 2 discrete peaks/bands were observed. (b) Monoclonal PCR products give rise to homoduplexes (second and fourth lane) whereas, polyclonal PCR products from heteroduplexes result in a smear of slow migrating fragments (third lane).

Table 12. TCRγ gene rearrangements in cHL grouped by patients age

Groups	n. of cases	TCRγ		
		Monoclonal	Oligoclonal	Polyclonal
cHL-old 50-69y	13	2 15%	2 15%	9 70%
cHL-old ≥70y	8	3 37%	0 -	5 63%
cHL-child 3-5y	13	2 15%	6 46%	5 39%
cHL-child 6-13y	25	8 32%	12 48%	5 20%
cHL-young 17-32y	10	0 -	0 -	10 100%

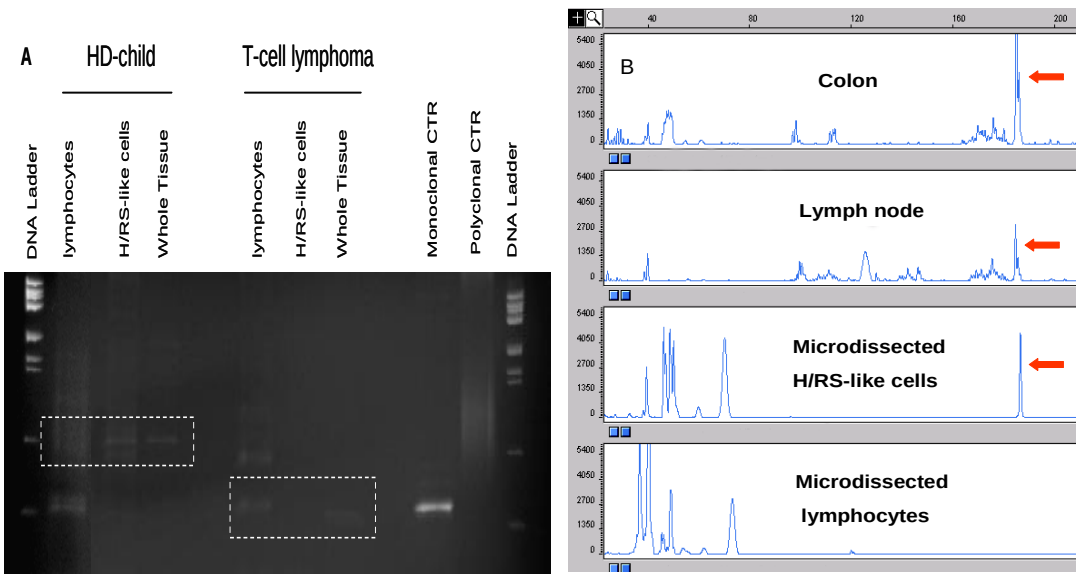

Figure 7. **Single cell analysis of TCRγ gene rearrangement in a cHL of childhood (A), and in a mucocutaneous ulcer of an old patient (B). A**. Eteroduplex analysis showed an identical-sized band in the microdissected H/RS-like cells and in the whole lymph node of an Iraqi children with EBV+HL; a smear was observed in microdissected sourrounding lymphocytes. In contrast, neoplastic lymphocytes and not the H/RS-like cells microdissected from an angioimmunoblastic T-cell lymphoma showed a band of the same size of that found in the whole biopsy sample. **B.** Gene Scan analysis of TCRγ clonality in an EBV+ mucocutaneous ulcer involving the sigmoid colon and the lymph nodes of an old Italian adult. A similar peak was observed in the whole tissue section of the colon, in the lymph node and in the microdissected H/RS-like cells but not in the surrounding lymphocytes.

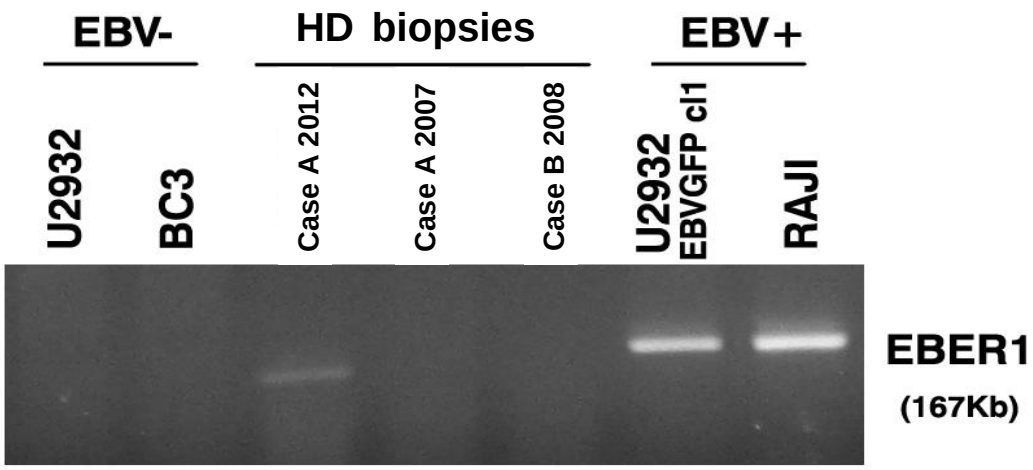

Figure 8. **PCR analysis for the detection of EBER-1 gene in methacronous lymphomas of two patients (case A and case B).** For case A the DNA extracted from the nodal HL diagnosed in 2012 was also included in the test. The analysis confirmed the presence of EBV-infection in the nodal HL of case A, and its absence in the prior extranodal non-Hodgkin B-cell lymphomas of both case A and case B.

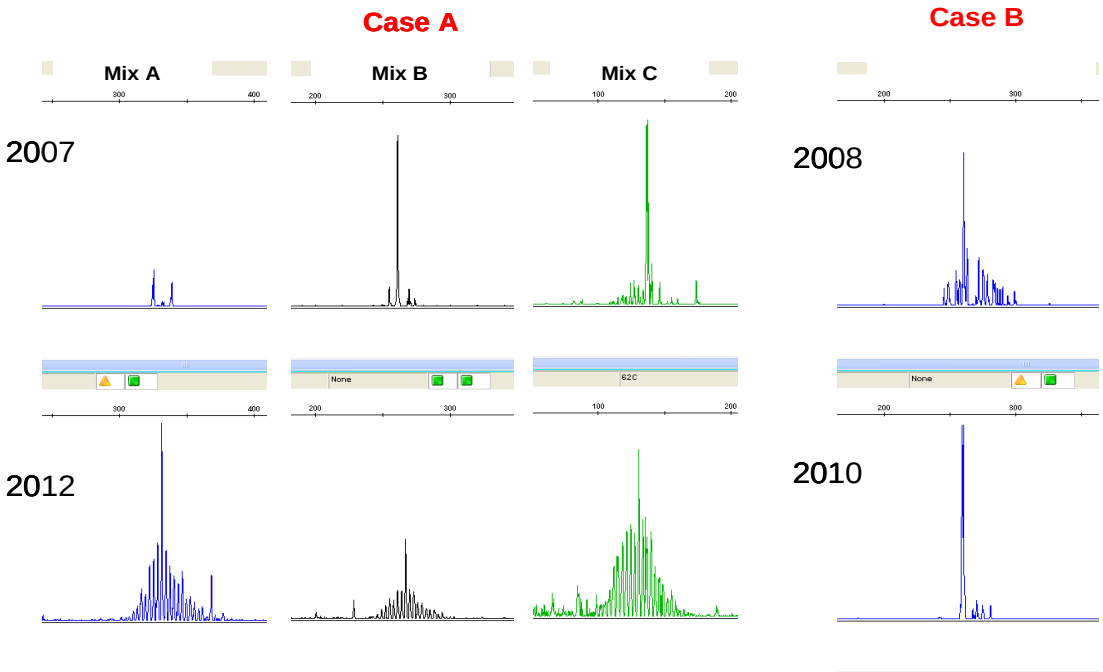

Figure 9. GeneScan analysis of IGH gene rearrangements in two adult patients with metachronous lymphomas. In **case A** IGH clonality was very similar between the metachronous tumors; a slightly difference in the positition of the monoclonal pick consisting in 3 base pairs was observed in all the three mastermix used for the analysis. In **case B** the two lymphomas showed an identical IGH rearrangement pattern, as shown in the representative images of one of the mastermix.

DISCUSSION

cHL in Iraqi children under 6 years of age is strictly associated with EBV-infection, MC-subtype and a cytotoxic background

In 1997 Glaser et al (*Glaser et al 1997*) published a meta-analysis of the epidemiologic characteristics of EBV-associated HL based on international data concerning 1546 HL patients of any age. They reported the existence of a higher risk of EBV+ cHL in children for both NS and MC subtypes; it was suggested that timing of infection greatly affects the association of EBV with cHL in children, with early age at infection strongly predicting EBV+ disease for both these histological subtypes. Our findings are consistent with this interpretation. In fact, we have observed that EBV infection of H/RS cells is present in 97% MC-HL and in 63% NS-HL of Iraqi children, whereas EBV+ NS in western countries accounts for approximately 10-25% of cases.

Table 13. EBV+ classic Hodgkin lymphoma in 300 Asian children in relation to age

Age range	Iraq		China[a]		India[b]		Total cases	EBV+ cases
	n. of cases	EBV+ cases	n. of cases	EBV+ cases	n. of cases	EBV+ cases		
3-5 y	18 35% [c]	18 100% [d]	33 32%	32 97%	17 12%	17 100%	68 23%	67 99%
6-10 y	24 47%	20 83%	58 56%	54 93%	93 64%	89 96%	175 58%	163 93%
11-14 y	9 18%	6 67%	13 12%	7 54%	35 24%	28 80%	57 19%	41 72%
Total cases	51	44 86%	104	93 89%	145	134 92%	300	271 90%

[a]Zhou XG et al. Cancer 2001; 92:1621-1631

[b]Dinand V et al. Eur. J. Cancer 2007 ; 43 :161-168

[c]Percent of the total number of cHL cases

[d]Percent of EBV+ cases/total number of classic HL in the age-range

Moreover, we have noted that there is a progressive age-related decrease in the percentage of EBV+ cases (from 100% to 67%), and a switch in the histological types from MC to NS. The epidemiology of cHL in Iraq is similar to that reported for China (*Zhou et al 2001*) and India (*Dinand et al 2007*). When the data of the three Asian countries are pooled together for a total of 300 children under 14 years of age (Table 13), it becomes clear that virtually all cases (99%)

of cHL under 6 years of age were EBV+, and that the percentage of EBV+ cases significantly decreased to 72% in the 11-14 years age-range. At present there is no definitive explanation for these findings. EBV-infection is strictly associated with MC histology, and it was speculated that a defective immune response, due to immaturity of the immune system might represent one of the triggering events (*Harris et al, 1998*; *Barros et al, 2012*; *Barros et al, 2011*). This interpretation is supported by the observation that the inflammatory background of MC-HL in children under 10 years is profoundly different from that of older children with NS subtype. In particular, Barros *et al.* found that the inflammatory background of EBV+ MC-HL-cases in children under 10 years was characterised by the presence of an intense T cell infiltrate exhibiting a cytotoxic/Th1 profile, whereas in the NS subtype of older children and in EBV-negative cases there is a higher number of CD4+ T cells with a more regulatory/Th2 profile (*Barros et al, IJC 2011).* In keeping with Barros *et al,* we found that in our series of pediatric cHL the number of GrB+ CTL cells was significantly higher in MC subtypes, in EBV-positive cases, and in patients of 3-5 years old.

LMP-1 gene polymorphism is not a frequent feature of EBV+ cHL of Iraqi children

EBV LMP-1 polymorphisms have been reported to have a greater tumorigenic potential than wild type LMP-1 gene. Among these a characteristic 30 basepair deletion in the 3' end of EBV LMP-1 gene has been found to be essential for the transformation activity of LMP-1 oncogene in transfection studies. The 30-bp deletion has been demonstrated in 4/12 (33%) EBV+ cHL cases from USA and in 12/26 (46%) cases from Brazil (*Hayashi et al 1997*). An higher incidence of EBV LMP-1 deletion has been found in HL occurring in HIV patients (89%) (*Docetti et al 1997*; *Bellas et al 1996*) and in Spanish children (79%) (*Santon et al 1998*). In our series of EBV+ HL of Iraqi children, we have found the presence of the 30-bp LMP-1 gene deletion in 32% of the cases. Our data are similar to what previously reported in USA and Brazil but dramatically different to that observed among Spanish children. It may be possible that geographical and ethnical factors may influence the incidence of EBV infection with EBV strains carrying distinct LMP-1 genes.

Aging is associated to the development of an EBV+ MC-HL subtype

Epidemiological studies reported that EBV+ HL of the mixed cellularity type shows a peak in older adults (>= 55 years) in developed countries (*Armstrong et al, 1998, Jarrett et al,*

2003; Jarrett et al, 2005). Consistently with these reports, we detected EBV infection in 16 of 30 cHL (53%) occurred in elderly Italian patients, with a significant predilection for MC than NS histology (82% vs 33%; p=0.036). Moreover, the percentage of EBV-associated cases, and of MC HL subtype gradually increased with advancing of patients age, supporting the hypothesis that EBV infection, histology and patients age are closely related. Jarrett *et al.* indicated that the decline in EBV-specific cellular immunity that occurs with age may be responsible of EBV reactivation in infected B-cells, thus increasing the risk of their malignant transformation (*Jarrett et al, 2005*). Ageing is known to be accompanied by thymic involution and also by a chronic low-grade inflammation state; these determine a progressive exhaustion of the CD8+ T-cell population and predispose elderly people to tumor development (*Vasto et al, 2007*; *Candore et al, 2010*). A major force able to drive a chronic pro-inflammatory state during aging may be represented by persistent infections by EBV and Cytomegalovirus virus (CMV). In support of this view in our series of cHL occurring in Italian old patients we observed a significant higher number of GrB+ CTL cells in the MC cases, which were found to be more commonly associated with EBV-infection.

cHL of elderly Italians retains higher expression of B-cell markers than cHL of Iraqi children

It is widely accepted that H/RS derive from mature B cells, although they show global loss of the B-cell phenotype. Mechanisms causing this extensive reprogramming of the tumor cells are mostly unknown. One of the contributing factor identified is given by the downregulation of the expression of several transcriptor factors in H/RS cells, including OCT2 and BOB1, that regulates the expression of many B-cell specific genes. An exception of this rule is represented by PAX5, the main B-cell lineage commitment factor. In fact, although PAX5 is found to be expressed in H/RS, many of its target genes are found to be down regulated, suggesting that its activity might be somehow impaired (*Saez et al, 2002; Kuppers, Hematol 2009*; *Browne et al, 2006*). Our data are consistent with what previously reported in the literature; in the series of Hodgkin lymphomas we investigated H/RS cells were usually positive for PAX5, and exhibited downregulation of the other B-cell markers CD20, CD79a, OCT2 and BOB1. However, when stratified for histology and patients age, our data showed that MC-HL of old Italian adults retain an higher expression of OCT-2 (73%) and BOB-1 (55%) compared to MC-HL of Iraqi children (OCT2 31%; BOB1 11%). The expression of OCT2 and BOB1 in elderly MC-HL was also greater than that reported in the literature (OCT-

2 12-63%; BOB-1 25-37%) (*Cosio et al, 2004*; *Browne et al, 2006*). This difference may be due to the fact that in previous studies HL of different subtypes or from patients with different age were pooled together, whereas in our study we sub-group HL cases by histology and patient age. Moreover, we considered both strong and weak, but clearly positive, staining patterns, whereas others may have considered as positive only strong intensity staining. Alternatively, our findings may raise the possibility that some of the elderly MC-HL are instead EBV+ DLBCL of the elderly. However, not all the HL of old adults that express OCT-2 and/or BOB-1 were EBV+. Furthermore, the consistent expression of CD15, together with the infrequent positivity for CD20 or CD79a, on morphologically typical H/RS cells argues against this hypothesis. Finally, Adams *et al.*, have found that BOB-1, OCT-2, CD79a and CD20 were more commonly expressed in post-transplant Hodgkin lymphoma (ptHL) versus cHL, suggesting that ptHL and cHL are closely related but not identical neoplasm, differing in the strict association with EBV infection, persistent phenotipic B-cell signature, and high expression of PI3K as well as the slightly CD4-depleted but TIA-1/Granzyme B-enriched cellular background composition in ptHL (*Adams et al, 2009*).

Restricted TCRγ gene rearrangements are a distinctive feature of cHL of Iraqi children

We have found a monoclonal IGH gene rearrangement in 37% of pediatric HL and in 48% of elderly cases using the BIOMED2 system applied to total DNA extracted from whole paraffin sections. This percentage is similar to that reported by Chute *et al.* (*Chute et al, 2008*) who could demonstrate IGH monoclonality in 24% of cHL cases, and borderline clonality in 17%.

An interesting finding of our study is the demonstration of monoclonal/oligoclonal TCRγ, suggestive of restricted T cell responses, in a large proportion of pediatric cHL (74%). This percentage is considerably higher than that reported for France, where only 13 of 85 (15%) cHL cases exhibited clonal TCRγ gene rearrangements (*Al Saati et a,l 1997*). However, the age of the French patients was not reported in the paper, so that it can not be excluded the existence of a possible association between patient's age and TCRγ gene status.

The biological significance of restricted T cell responses in HL involved tissues may be only matter of speculation. Restricted T cell responses against EBV antigens may be probably indicative of a poorly efficient immune response. In fact, during primary EBV infection, recovery from the symptoms is coincident with broad T cell reactivity to multiple epitopes whereas, in patients with a delayed healing a narrowly focused response has been observed

(*Bharadwaj et al, 2001)*. More recently, it has been shown that a distinctive serologic response to EBV latent antigens, indicative of immune dysfunction, is associated with an increased risk to develop EBV+ cHL (*Levin et al, 2012*). Moreover, T cell restricted responses were previously observed in about 30% of EBV-driven lymphoproliferative disorders secondary to iatrogenic immunosuppression (*Au et al, 2006; Dojcinov et al, 2010*) and to immunosenescence owing to ageing (*Dojcinov et al, 2011*). In this latter condition T cell responses to viruses are profoundly affected due to accumulation of mature CD8+ T cells with diminished functionality. A similar condition was described in early infancy, where cytotoxic T lymphocyte responses to viral infections remain low as compared with adults (*Kovarik et al, 1998*). Furthermore, we detected a consistent polyclonal TCRγ rearrangements in 10 cases of EBV-negative NS-HL of Italian young adults. This supports the hypothesis that high incidence of T cell restricted responses in cHL of childhood might be the expression of a defective local immune response, which might be particularly pronounced in children under 14 years of age.

In contrast to Iraqi children, our series of cHL occurring in old Italian adults showed monoclonal/oligoclonal TCRγ rearrangements only in 33% of the cases. This difference might be related to a different tumor microenviroment. It is possible that children and older adults differ in their response to the tumor not only with respect to the recruited cell types but also to the functional status of these cells. Barros *et al.* have recently found that tumor microenviroment composition in pediatric cHL is distinct from adults, with a higher number of CD14+ cells and of CD3+, CD8+, TIA-1+, and TBET+ lymphocytes in children under 10 years (*Barros et al, IJC 2011; Barros et al, 2012*). In our series of pediatric and elderly cHL we did not find any significant difference in the number of CD3+, CD4+, CD8+, CD56+, GrB+ cells, with both pediatric and elderly cHL, having a more cytotoxic background in MC as compared to NS subtype. Neverthless, we have no data on the functionality of these cells to exclude that different TCRγ clonality pattern may reflect diverse states of immune responsiveness.

Concomitant IGH and TCRγ rearrangements occurrence in H/RS cells

A small proportion (10-14%) of HL cases from both pediatric and elderly patients showed a concomitant IGH and TCRγ clonality. Dual IGH and TCRγ clonality has been previously reported by Saati *et al.* in 6% of a series of 85 HL. Reports on single cell analysis of IGH and TCRγ rearrangements in HL showed that clonality of IGH and TCRγ genes in H/RS cells are mutually exclusive, with evidence of TCR genes clonality in H/RS cells expressing T-

cell markers (*Seitz et al, 2000*; *Müschen et al, 2000*). Thanks to laser capture micro-dissection we provide evidence that TCRγ clonal rearrangement may occur in neoplastic B cells with H/RS-like morphology lacking expression of T-cell associated markers. Thus, our findings raise the possibility that in H/RS cells a still unrecognized transforming event might cause deregulation/impairment of the RAG (Recombination Activating Gene) enzyme system inducing TCR gene rearrangements in B cells which have already undergone IGH rearrangements. A similar pathogenetic mechanism has already been proposed to explain the presence of concomitant IGH and TCR rearrangements in a consistent number of cases of precursor-B-ALL (*Szczepański et al, 1999*).

Composite EBV+ HL and EBV-negative non-Hodgkin B cell-lymphoma

It is interesting to note that in our series of elderly EBV+ HL two patients had a prior history of an EBV-negative extranodal non-Hodgkin B-cell lymphoma. GeneScan analysis demonstrated an identical monoclonal IgH rearrangement in the methachronous lymphomas of one of the two patients, suggesting derivation from the same neoplastic clone. In the other patient, a very similar IgH clonality pattern was observed between the two lymphomas. Although IGH somatic mutation seems not to be a characteristic feature of cHL (*Kuppers et al, 1994*), in this patient it can be possible that additional mutations in the rearranged IGH gene may have occurred in the subsequent tumor. Alternatively, it might be possible that the two tumors represent unrelated neoplasms. To address this question, we are presently undergoing IGH mutational analysis of the methachronous lymphomas. Recently, a single cell analysis have been conduct to investigate the clonal relationship of a series of cHL and its recurrences (*Oberman et al, 2011*). The authors found both related and unrelated relapses, and observed an EBV-association switch in a proportion of the clonally unrelated HL. Similarly, Tinguely *et al*. described a composite lymphoma consisting of a mantle cell lymphoma (MCL) and an EBV+ HL, in which single cell analysis of rearranged V genes revealed a clonal relationship between the two lymphomas. Moreover, the authors detected somatic mutations in H/RS but not in MCL cells, and noticed that only a subclone of the H/RS population, with a particular mutation pattern in the V genes, was EBV-infected. This finding represented for Tinguely *et al* a strong indication that EBV infection of the H/RS cell precursor occurred in the germinal center (GC) (*Tinguely et al, 2003*). The identification of composite synchronous and methachronous tumors suggests the possibility of the existence of a common tumor stem or precursor cell that already carried some transforming events. Nevertheless, it might be possible that unknown genetic or

environmental factors (i.e. chemotherapies, aging) predispose an individual to the development of clonally unrelated lymphomas.

The presence of EBV exclusively in the relapsed HL in our cases and in the ones described in the literature (*Oberman et al, 2011*) raises concern about the role of EBV in the pathogenesis of the disease. It can be speculated that a common precursor might be subsequentially infected by EBV during the remission period between the two methachronous lymphomas, probably through virions released by non-neoplastic memory B cells. Alternatively, based on the "hit and run" theory, we could assume that EBV infection occurred early during tumorigenesis, and that only tumor cells with a defective EBV genome that loss the virus might have had a selective advantage in the prior lymphoma (*Jox et al, 1997; Trivedi et al, 1995*). However, this theory is difficult to prove because we and others have failed to find any trace of previous EBV infection in H/RS cells of EBV-negative lymphomas (*Staratschek-Jox et al, 2000*). In contrast, arguments in favour of the theory are the observation that EBV+ HL cases may relapse as EBV-negative cases (*Delecluse et al, 1997; Nerurkar et al, 2000*), the notion that infectious mononucleosis represents a risk factor also for the development of EBV-negative HL (*Jarrett et al, 2002*; *Sleckman et al, 1*998), and the demonstration of defective forms of EBV which progressively lose the ability to infect host cells (*Gan et al, 2002*).

In conclusion, our data suggest that cHL occurring in Iraqi children and in Italian older adults are very similar with an age-depend variation in histology and EBV-infection, suggesting that in both class of patients an impaired immune system may be critical for the development of the disease. However, significant differences in B-cell phenotype of H/RS cells and in pattern of TCRγ gene rearrangements suggest that cHL of pediatric and elderly patients are closely related but not identical neoplasm, probably representing a continuum spectrum.

REFERENCES

Adams H, Campidelli C, Dirnhofer S, Pileri SA, Tzankov A. Clinical, phenotypic and genetic similarities and disparities between post-transplant and classical Hodgkin lymphomas with respect to therapeutic targets. Expert Opin Ther Targets. 2009 Oct;13(10):1137-45.

Aldinucci D, Lorenzon D, Cattaruzza L, Pinto A, Gloghini A, Carbone A, Colombatti A. Expression of CCR5 receptors on Reed-Sternberg cells and Hodgkin lymphoma cell lines: involvement of CCL5/Rantes in tumor cell growth and microenvironmental interactions. Int J Cancer. 2008 Feb 15;122(4):769-76.

Aldinucci D, Gloghini A, Pinto A, De Filippi R, Carbone A. The classical Hodgkin's lymphoma microenvironment and its role in promoting tumour growth and immune escape. J Pathol. 2010 Jul;221(3):248-63.

Al Saati T, Galoin S, Gravel S, et al. IgH and TcR-gamma gene rearrangements identified in Hodgkin's disease by PCR demonstrate lack of correlation between genotype, phenotype, and Epstein-Barr virus status. J Pathol 1997:181:387-393.

Alvaro T, Lejeune M, Salvadó MT, Bosch R, García JF, Jaén J, Banham AH, Roncador G, Montalbán C, Piris MA. Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. Clin Cancer Res. 2005 Feb 15;11(4):1467-73.

Ambinder RF. Gammaherpesviruses and "Hit-and-Run" oncogenesis. Am J Pathol. 2000 Jan;156(1):1-3. Review.

Anderton E, Yee J, Smith P, Crook T, White RE, Allday MJ. Two Epstein-Barr virus (EBV) oncoproteins cooperate to repress expression of the proapoptotic tumour-suppressor Bim: clues to the pathogenesis of Burkitt's lymphoma. Oncogene. 2008 Jan 17;27(4):421-33. Epub 2007 Jul 23.

Armstrong AA, Alexander FE, Cartwright R, Angus B, Krajewski AS, Wright DH, Brown I, Lee F, Kane E, Jarrett RF. Epstein-Barr virus and Hodgkin's disease: further evidence for the three disease hypothesis. Leukemia. 1998 Aug;12(8):1272-6

Asano N, Yamamoto K, Tamaru J, Oyama T, Ishida F, Ohshima K, Yoshino T, Nakamura N, Mori S, Yoshie O, Shimoyama Y, Morishima Y, Kinoshita T, Nakamura S. Age-related Epstein-Barr virus (EBV)-associated B-cell lymphoproliferative disorders: comparison with EBV-positive classic Hodgkin lymphoma in elderly patients. Blood. 2009 Mar 19;113(12):2629-36.

Au WY, Ma ES, Choy C, et al. Therapy-related lymphomas in patients with autoimmune diseases after treatment with disease-modifying anti-rheumatic drugs. Am J Hematol 2006: 81:5-11.

Bajaj BG, Murakami M, Robertson ES. Molecular biology of EBV in relationship to AIDS-associated oncogenesis. Cancer Treat Res 2007;133:141–62.

Barros MH, Hassan R, Niedobitek G. Disease patterns in pediatric classical Hodgkin lymphoma: a report from a developing area in Brazil. Hematol Oncol. 2011 Dec;29(4):190-5.

Barros MH, Vera-Lozada G, Soares FA, Niedobitek G, Hassan R. Tumor microenvironment composition in pediatric classical Hodgkin lymphoma is modulated by age and Epstein-Barr virus infection. Int J Cancer. 2011 Sep 1;131(5):1142-52.

Barros MH, Hassan R, Niedobitek G. Tumor-associated macrophages in pediatric classical Hodgkin lymphoma: association with Epstein-Barr virus, lymphocyte subsets, and prognostic impact. Clin Cancer Res. 2012 Jul 15;18(14):3762-71.

Baumforth KR, Birgersdotter A, Reynolds GM, Wei W, Kapatai G, Flavell JR, Kalk E, Piper K, Lee S, Machado L, Hadley K, Sundblad A, Sjoberg J, Bjorkholm M, Porwit AA, Yap LF, Teo S, Grundy RG, Young LS, Ernberg I, Woodman CB, Murray PG. Expression of the Epstein-Barr virus-encoded Epstein-Barr virus nuclear antigen 1 in Hodgkin's lymphoma cells mediates Up-regulation of CCL20 and the migration of regulatory T cells. Am J Pathol. 2008 Jul;173(1):195-204.

Bechtel D, Kurth J, Unkel C, Küppers R. Transformation of BCR-deficient germinal-center B cells by EBV supports a major role of the virus in the pathogenesis of Hodgkin and posttransplantation lymphomas. Blood. 2005 Dec 15;106(13):4345-50. Epub 2005 Aug 30.

Bellas C, Santón A, Manzanal A, Campo E, Martin C, Acevedo A, Varona C, Forteza J, Morente M, Montalbán C. Pathological, immunological, and molecular features of Hodgkin's disease associated with HIV infection. Comparison with ordinary hodgkin's disease. Am J Surg Pathol. 1996 Dec;20(12):1520-4.

Bharadwaj M, Burrows SR, Burrows JM, el al. Longitudinal dynamics of antigen-specific CD8+ cytotoxic T lymphocytes following primary Epstein-Barr virus infection. Blood 2001: 98:2588-2589.

Browne P, Petrosyan K, Hernandez A, Chan JA. The B-cell transcription factors BSAP, Oct-2, and BOB.1 and the pan-B-cell markers CD20, CD22, and CD79a are useful in the differential diagnosis of classic Hodgkin lymphoma. Am J Clin Pathol. 2003 Nov;120(5):767-77.

Candore G, Caruso C, Jirillo E, Magrone T, Vasto S. Low grade inflammation as a common pathogenetic denominator in age-related diseases: novel drug targets for anti-ageing strategies and successful ageing achievement. Curr Pharm Des. 2010;16(6):584-96.

Carbone A, Gloghini A, Gruss HJ, Pinto A. CD40 ligand is constitutively expressed in a subset of T cell lymphomas and on the microenvironmental reactive T cells of follicular lymphomas and Hodgkin's disease. Am J Pathol. 1995;147(4):912–922.

Carbone A, Gloghini A, Dotti G. EBV-associated lymphoproliferative disorders: classification and treatment. Oncologist. 2008 May;13(5):577-85.

Carbone A, Spina M, Gloghini A, Tirelli U. Classical Hodgkin's lymphoma arising in different host's conditions: pathobiology parameters, therapeutic options, and outcome. Am J Hematol. 2011 Feb;86(2):170-9.

Chaganti S, Bell AI, Pastor NB, Milner AE, Drayson M, Gordon J, Rickinson AB. Epstein-Barr virus infection in vitro can rescue germinal center B cells with inactivated immunoglobulin genes. Blood. 2005 Dec 15;106(13):4249-52.

Chapman CJ, Zhou JX, Gregory C, Rickinson AB, Stevenson FK. VH and VL gene analysis in sporadic Burkitt's lymphoma shows somatic hypermutation, intraclonal heterogeneity, and a role for antigen selection. Blood. 1996 Nov 1;88(9):3562-8.

Chemnitz JM, Eggle D, Driesen J, Classen S, Riley JL, Debey-Pascher S, et al . RNA fingerprints provide direct evidence for the inhibitory role of TGFβ and PD-1 on CD4+ T cells in Hodgkin lymphoma. Blood 2007; 110: 3226–3233.

Chetaille B, Bertucci F, Finetti P, Esterni B, Stamatoullas A, Picquenot JM, Copin MC, Morschhauser F, Casasnovas O, Petrella T, Molina T, Vekhoff A, Feugier P, Bouabdallah R, Birnbaum D, Olive D, Xerri L. Molecular profiling of classical Hodgkin lymphoma tissues uncovers variations in the tumor microenvironment and correlations with EBV infection and outcome. Blood. 2009 Mar 19;113(12):2765-3775.

Chute DJ, Cousar JB, Mahadevan MS, el al. Detection of immunoglobulin heavy chain gene rearrangements in classic Hodgkin lymphoma using commercially available BIOMED-2 primers. Diagn Mol Pathol 2008: 17:65-72

Cohen JI. Epstein-Barr virus infection. N Engl J Med.2000;643(7):481-492

Cohen JI, Bollard CM, Khanna R, Pittaluga S. Current understanding of the role of Epstein-Barr virus in lymphomagenesis and therapeutic approaches to EBV-associated lymphomas. Leuk Lymphoma. 2008;49 Suppl 1:27-34.

Dalla-Favera R, Bregni M, Erikson J, Patterson D, Gallo RC, Croce CM. Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci U S A. 1982 Dec;79(24):7824-7.

Dallenbach FE, Stein H. Expression of T-cell-receptor beta chain in Reed-Sternberg cells. Lancet. 1989 Oct 7;2(8667):828-30.

Davis JE, Sherritt MA, Bharadwaj M, Morrison LE, Elliott SL, Kear LM, Maddicks-Law J, Kotsimbos T, Gill D, Malouf M, Falk MC, Khanna R, Moss DJ. Determining virological, serological and immunological parameters of EBV infection in the development of PTLD. Int Immunol. 2004 Jul;16(7):983-9.

Delecluse HJ, Marafioti T, Hummel M, Dallenbach F, Anagnostopoulos I, Stein H. Disappearance of the Epstein-Barr virus in a relapse of Hodgkin's disease. J Pathol. 1997;182:475-9.

De Re V, Boiocchi M, De Vita S, Dolcetti R, Gloghini A, Uccini S, Baroni C, Scarpa A, Cattoretti G, Carbone A. Subtypes of Epstein-Barr virus in HIV-1-associated and HIV-1-unrelated Hodgkin's disease cases. Int J Cancer. 1993 Jul 30;54(6):895-8.

Dinand V, Dawar R, Arya LS, et al. Hodgkin's lymphoma in Indian children: prevalence and significance of Epstein-Barr virus detection in Hodgkin's and Reed-Sternberg cells. Eur J Cancer 2007: 43:161-168.

Di Napoli A, Giubettini M, Duranti E, Ferrari A, Guglielmi C, Uccini S, Ruco L. Iatrogenic EBV-positive lymphoproliferative disorder with features of EBV+ mucocutaneous ulcer: evidence for concomitant TCRγ/IGH rearrangements in the Hodgkin-like neoplastic cells. Virchows Arch. 2011 May;458(5):631-6.

Dojcinov SD, Venkataraman G, Raffeld M, et al. EBV positive mucocutaneous ulcer-A study of 26 cases associated with various sources. Am J Surg Pathol 2010: 34:405-417.

Dojcinov SD, Venkataraman G, Pittaluga S, et al. Age-related EBV-associated lymphoproliferative disorders in the Western population: a spectrum of reactive lymphoid hyperplasia and lymphoma. Blood 2011: 117:4726-4735.

Dolcetti R, Zancai P, De Re V, Gloghini A, Bigoni B, Pivetta B, De Vita S, Carbone A, Boiocchi M. Epstein-Barr virus strains with latent membrane protein-1 deletions: prevalence in the Italian population and high association with human immunodeficiency virus-related Hodgkin's disease. Blood. 1997 Mar 1;89(5):1723-31.

D'Souza BN, Edelstein LC, Pegman PM, Smith SM, Loughran ST, Clarke A, et al. Nuclear factor kappa B-dependent activation of the antiapoptotic bfl-1 gene by the Epstein-Barr virus latent membrane protein 1 and activated CD40 receptor. J Virol 2004;78:1800–16.

Dutton A, Reynolds GM, Dawson CW, Young LS, Murray PG. Constitutive activation of phosphatidyl-inositide 3 kinase contributes to the survival of Hodgkin's lymphoma cells through a mechanism involving Akt kinase and mTOR. J Pathol. 2005;205(4):498–506.

Farrell K, Jarrett RF. The molecular pathogenesis of Hodgkin lymphoma. Histopathology. 2011 Jan;58(1):15-25.

Fischer M, Juremalm M, Olsson N, Backlin C, Sundström C, Nilsson K, Enblad G, Nilsson G. Expression of CCL5/RANTES by Hodgkin and Reed-Sternberg cells and its possible role in the recruitment of mast cells into lymphomatous tissue. Int J Cancer. 2003 Nov 1;107(2):197-201.

Flavell JR, Baumforth KR, Wood VH, Davies GL, Wei W, Reynolds GM, Morgan S, Boyce A, Kelly GL, Young LS, Murray PG. Down-regulation of the TGF-beta target gene, PTPRK, by the Epstein-Barr virus encoded EBNA1 contributes to the growth and survival of Hodgkin lymphoma cells. Blood. 2008 Jan 1;111(1):292-301.

Gan YJ, Razzouk BI, Su T, Sixbey JW. A defective, rearranged Epstein-Barr virus genome in EBER-negative and EBER-positive Hodgkin's disease. Am J Pathol. 2002 Mar;160(3):781-6.

Gallagher A, Perry J, Freeland J, Alexander FE, Carman WF, Shield L, Cartwright R, Jarrett RF. Hodgkin lymphoma and Epstein-Barr virus (EBV): no evidence to support hit-and-run mechanism in cases classified as non-EBV-associated. Int J Cancer. 2003 May 1;104(5):624-30.

Gandhi MK, Moll G, Smith C, Dua U, Lambley E, Ramuz O, et al . Galectin-1-mediated suppression of Epstein–Barr virus specific T-cell immunity in classic Hodgkin lymphoma. Blood 2007; 110: 1326–1329.

García-Cosío M, Santón A, Martín P, Camarasa N, Montalbán C, García JF, Bellas C. Analysis of transcription factor OCT.1, OCT.2 and BOB.1 expression using tissue arrays in classical Hodgkin's lymphoma. Mod Pathol. 2004 Dec;17(12):1531-8.

Gibson SE, Hsi ED. Epstein-Barr virus-positive B-cell lymphoma of the elderly at a United States tertiary medical center: an uncommon aggressive lymphoma with a nongerminal center B-cell phenotype. Hum Pathol. 2009 May;40(5):653-61.

Glaser SL, Lin RJ, Stewart SL, et al. Epstein-Barr virus-associated Hodgkin's disease: epidemiologic characteristics in international data. Int J Cancer 1997: 70:375-382.

Gulley ML, Eagan PA, Quintanilla-Martinez L, Picado AL, Smir BN, Childs C, Dunn CD, Craig FE, Williams JW Jr, Banks PM. Epstein-Barr virus DNA is abundant and monoclonal in the Reed-Sternberg cells of Hodgkin's disease: association with mixed cellularity subtype and Hispanic American ethnicity. Blood. 1994 Mar 15;83(6):1595-602.

Harris NL. The many faces of Hodgkin's disease around the world: what have we learned from its pathology? Ann Oncol 1998: 9 :45-56.

Hayashi K, Chen WG, Chen YY, et al. Deletion of Epstein-Barr virus latent membrane protein 1 gene in United States and Brazilian Hodgkin's disease and reactive lymphoid tissue: high frequency of a 30-bp deletion. Hum Pathol 1997:28:1408-1414.

Henle G, Henle W, Diehl V. Relation of a Burkitt tumor associated herpes-type virus to infectious mononucleosis. Proc Natl Acad Sci USA 1968; 59: 94–101.

Heslop HE. How I treat EBV lymphoproliferation. Blood. 2009 Nov 5;114(19):4002-8.

Hislop AD, Taylor GS, Sauce D, Rickinson AB. Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. Annu Rev Immunol. 2007;25:587–617

Hjalgrim H, Rostgaard K, Johnson PC, Lake A, Shield L, Little AM, Ekstrom-Smedby K, Adami HO, Glimelius B, Hamilton-Dutoit S, Kane E, Taylor GM, McConnachie A, Ryder LP, Sundstrom C, Andersen PS, Chang ET, Alexander FE, Melbye M, Jarrett RF. HLA-A alleles and infectious mononucleosis suggest a critical role for cytotoxic T-cell response in EBV-related Hodgkin lymphoma. Proc Natl Acad Sci U S A. 2010 Apr 6;107(14):6400-5.

Hoeller S, Tzankov A, Pileri SA, Went P, Dirnhofer S. Epstein-Barr virus-positive diffuse large B-cell lymphoma in elderly patients is rare in Western populations. Hum Pathol. 2010 Mar;41(3):352-7.

Hu LF, Chen F, Zheng X, Ernberg I, Cao SL, Christensson B, Klein G, Winberg G. Clonability and tumorigenicity of human epithelial cells expressing the EBV encoded membrane protein LMP1. Oncogene. 1993 Jun;8(6):1575-83.

Huang X, Nolte I, Gao Z, Vos H, Hepkema B, Poppema S, van den Berg A, Diepstra A. Epidemiology of classical Hodgkin lymphoma and its association with Epstein Barr virus in Northern China. PLoS One. 2011;6(6):e21152.

Hummel M, Bentink S, Berger H, Klapper W, Wessendorf S, Barth TF, Bernd HW, Cogliatti SB, Dierlamm J, Feller AC, Hansmann ML, Haralambieva E, Harder L, Hasenclever D, Kühn M, Lenze D, Lichter P, Martin-Subero JI, Möller P, Müller-Hermelink HK, Ott G, Parwaresch RM, Pott C, Rosenwald A, Rosolowski M, Schwaenen C, Stürzenhofecker B, Szczepanowski M, Trautmann H, Wacker HH, Spang R, Loeffler M, Trümper L, Stein H, Siebert R; Molecular Mechanisms in Malignant Lymphomas Network Project of the Deutsche Krebshilfe. A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. N Engl J Med. 2006 Jun 8;354(23):2419-30.

Jarrett RF. Viruses and Hodgkin's lymphoma. Ann Oncol. 2002;13 Suppl 1:23-9.

Jarrett RF, Krajewski AS, Angus B, Freeland J, Taylor PR, Taylor GM, Alexander FE. The Scotland and Newcastle epidemiological study of Hodgkin's disease: impact of histopathological review and EBV status on incidence estimates. J Clin Pathol. 2003 Nov;56(11):811-6. Erratum in: J Clin Pathol. 2004 Jan;57(1):112.

Jarrett RF. Risk factors for Hodgkin's lymphoma by EBV status and significance of detection of EBV genomes in serum of patients with EBV-associated Hodgkin's lymphoma. Leuk Lymphoma. 2003;44 Suppl 3:S27-32.

Jarrett RF, Stark GL, White J, Angus B, Alexander FE, Krajewski AS, Freeland J, Taylor GM, Taylor PR; Scotland and Newcastle Epidemiology of Hodgkin Disease Study Group. Impact of tumor Epstein-Barr virus status on presenting features and outcome in age-defined subgroups of patients with classic Hodgkin lymphoma: a population-based study. Blood. 2005 Oct 1;106(7):2444-51.

Johannsen E, Koh E, Mosialos G, Tong X, Kieff E, Grossman SR. Epstein-Barr virus nuclear protein 2 transactivation of the latent membrane protein 1 promoter is mediated by J kappa and PU.1. J Virol. 1995 Jan;69(1):253-62.

Joos S, Küpper M, Ohl S, von Bonin F, Mechtersheimer G, Bentz M, Marynen P, Möller P, Pfreundschuh M, Trümper L, Lichter P. Genomic imbalances including amplification of the tyrosine kinase gene JAK2 in CD30+ Hodgkin cells. Cancer Res. 2000;60(3):549–552.

Jox A, Rohen C, Belge G, Bartnitzke S, Pawlita M, Diehl V, Bullerdiek J, Wolf J. Integration of Epstein-Barr virus in Burkitt's lymphoma cells leads to a region of enhanced chromosome instability. Ann Oncol. 1997;8 Suppl 2:131-5.

Kanno H, Yasunaga Y, Iuchi K, Yamauchi S, Tatekawa T, Sugiyama H, Aozasa K. Interleukin-6-mediated growth enhancement of cell lines derived from pyothorax-associated lymphoma. Lab Invest. 1996 Aug;75(2):167-73.

Kanno H, Naka N, Yasunaga Y, Iuchi K, Yamauchi S, Hashimoto M, Aozasa K. Production of the immunosuppressive cytokine interleukin-10 by Epstein-Barr-virus-expressing pyothorax-associated lymphoma: possible role in the development of overt lymphoma in immunocompetent hosts. Am J Pathol. 1997 Jan;150(1):349-57.

Kanzler H, Küppers R, Hansmann ML, Rajewsky K. Hodgkin and Reed-Sternberg cells in Hodgkin's disease represent the outgrowth of a dominant tumor clone derived from (crippled) germinal center B cells. J Exp Med. 1996 Oct 1;184(4):1495-505.

Kaye KM, Izumi KM, Kieff E. Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. Proc Natl Acad Sci U S A. 1993 Oct 1;90(19):9150-4.

Kapatai G, Murray P. Contribution of the Epstein Barr virus to the molecular pathogenesis of Hodgkin lymphoma. J Clin Pathol. 2007 Dec;60(12):1342-9.

Kapp U, Wolf J, Hummel M, Pawlita M, von Kalle C, Dallenbach F, Schwonzen M, Krueger GR, Muller-Lantzsch N and Fonatsch C. Hodgkin's lymphoma-derived tissue serially transplanted into severe combined immunodeficient mice. Blood. 1993 Aug 15;82(4):1247-56.

Kapp U, Düx A, Schell-Frederick E, Banik N, Hummel M, Mücke S, Fonatsch C, Bullerdiek J, Gottstein C, Engert A, et al. Disseminated growth of Hodgkin's-derived cell lines L540 and L540cy in immune-deficient SCID mice. Ann Oncol. 1994;5 Suppl 1:121-6.

Kelley TW, Pohlman B, Elson P, Hsi ED. The ratio of FOXP3+ regulatory T cells to granzyme B+ cytotoxic T/NK cells predicts prognosis in classical Hodgkin lymphoma and is independent of bcl-2 and MAL expression. Am J Clin Pathol. 2007 Dec;128(6):958-65.

Khanna R, et al. Localization of Epstein-Barr virus cytotoxic T cell epitopes using recombinant vaccinia: implications for vaccine development. J Exp Med. 1992;176(1):169–176.

Kilger E, Kieser A, Baumann M, Hammerschmidt W. Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor. EMBO J. 1998 Mar 16;17(6):1700-9.

Kovarik J, Siegrist CA Immunity in early life. Immunol Today 1998: 19:150-152.

Kulwichit W, Edwards RH, Davenport EM, et al. Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice. Proc Natl Acad Sci U S A 1998;95:11963–11968.

Küppers R, Rajewsky K, Zhao M, Simons G, Laumann R, Fischer R, Hansmann ML. Hodgkin disease: Hodgkin and Reed-Sternberg cells picked from histological sections show clonal immunoglobulin gene rearrangements and appear to be derived from B cells at various stages of development. Proc Natl Acad Sci U S A. 1994 Nov 8;91(23):10962-6.

Küppers R. Molecular biology of Hodgkin lymphoma. Hematology Am Soc Hematol Educ Program. 2009:491-6.

Küppers R. The biology of Hodgkin's lymphoma. Nat Rev Cancer. 2009 Jan;9(1):15-27.

Küppers R, Engert A, Hansmann ML. Hodgkin lymphoma. J Clin Invest. 2012 Oct 1;122(10):3439-47.

Leen A, et al. Differential immunogenicity of Epstein-Barr virus latent-cycle proteins for human CD4(+) T-helper 1 responses. J Virol. 2001;75(18):8649–8659.

Levin LI, Chang ET, Ambinder RF, Lennette ET, Rubertone MV, Mann RB, Borowitz M, Weir EG, Abbondanzo SL, Mueller NE. Atypical prediagnosis Epstein-Barr virus serology restricted to EBV-positive Hodgkin lymphoma. Blood. 2012 Nov 1;120(18):3750-5. doi: 10.1182/blood-2011-12-390823.

Ma SD, Hegde S, Young KH, Sullivan R, Rajesh D, Zhou Y, Jankowska-Gan E, Burlingham WJ, Sun X, Gulley ML, Tang W, Gumperz JE, Kenney SC. A new model of Epstein-Barr virus infection reveals an important role for early lytic viral protein expression in the development of lymphomas. J Virol. 2011 Jan;85(1):165-77.

MacMahon B. Epidemiology of Hodgkin's disease. Cancer Res 1966; 26: 1189–1200.

Mancao C, Altmann M, Jungnickel B, Hammerschmidt W. Rescue of "crippled" germinal center B cells from apoptosis by Epstein-Barr virus. Blood. 2005 Dec 15;106(13):4339-44.

Mani H, Jaffe ES. Hodgkin lymphoma: an update on its biology with new insights into classification. Clin Lymphoma Myeloma. 2009 Jun;9(3):206-16.

Marshall NA, Christie LE, Munro LR, Culligan DJ, Johnston PW, Barker RN, Vickers MA. Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. Blood. 2004;103(5):1755–1762.

Martin-Subero JI, et al. Chromosomal breakpoints affecting immunoglobulin loci are recurrent in Hodgkin and Reed-Sternberg cells of classical Hodgkin lymphoma. Cancer Res. 2006;66(21):10332–10338.

Münz C, Bickham KL, Subklewe M, Tsang ML, Chahroudi A, Kurilla MG, Zhang D, O'Donnell M, Steinman RM. Human CD4(+) T lymphocytes consistently respond to the latent Epstein-Barr virus nuclear antigen EBNA1. J Exp Med. 2000;191(10):1649–1660.

Murray RJ, et al. Identification of target antigens for the human cytotoxic T cell response to Epstein-Barr virus (EBV): implications for the immune control of EBV-positive malignancies. J Exp Med.1992;176(1):157–168.

Müschen M, Rajewsky K, Bräuninger A, Baur AS, Oudejans JJ, Roers A, Hansmann ML, Küppers R. Rare occurrence of classical Hodgkin's disease as a T cell lymphoma. J Exp Med. 2000 Jan 17;191(2):387-94.

Nerurkar AY, Vijayan P, Srinivas V, Soman CS, Dinshaw KA, Advani SH, Magrath I, Bhatia K, Naresh KN. Discrepancies in Epstein-Barr virus association at presentation and relapse of classical Hodgkin's disease: impact on pathogenesis. Ann Oncol. 2000;11:475-8.

Niedobitek G, Agathanggelou A, Herbst H, Whitehead L, Wright DH, Young LS. Epstein-Barr virus (EBV) infection in infectious mononucleosis: virus latency, replication and phenotype of EBV-infected cells. J Pathol. 1997 Jun;182(2):151-9.

Nozawa Y, Wakasa H, Abe M. Costimulatory molecules (CD80 and CD86) on Reed-Sternberg cells are associated with the proliferation of background T cells in Hodgkin's disease. Pathol Int. 1998; 48(1):10–14.

Obermann EC, Mueller N, Rufle A, Menter T, Mueller-Garamvoelgyi E, Cathomas G, Dirnhofer S, Tzankov A. Clonal relationship of classical hodgkin lymphoma and its recurrences. Clin Cancer Res. 2011 Aug 15;17(16):5268-74.

Peh SC, Kim LH, Poppema S. TARC, a CC chemokine, is frequently expressed in classic Hodgkin's lymphoma but not in NLP Hodgkin's lymphoma, T-cell-rich B-cell lymphoma, and most cases of anaplastic large cell lymphoma. Am J Surg Pathol. 2001 Jul;25(7):925-9.

Pinkus GS, Pinkus JL, Langhoff E, Matsumura F, Yamashiro S, Mosialos G, Said JW. Fascin, a sensitive new marker for Reed-Sternberg cells of hodgkin's disease. Evidence for a dendritic or B cell derivation? Am J Pathol. 1997 Feb;150(2):543-62.

Portis T, Dyck P, Longnecker R. Epstein-Barr Virus (EBV) LMP2A induces alterations in gene transcription similar to those observed in Reed-Sternberg cells of Hodgkin lymphoma. Blood. 2003 Dec 1;102(12):4166-78.

Quintanilla-Martinez L, Fend F, Moguel LR, Spilove L, Beaty MW, Kingma DW, Raffeld M, Jaffe ES. Peripheral T-cell lymphoma with Reed-Sternberg-like cells of B-cell phenotype and genotype associated with Epstein-Barr virus infection. Am J Surg Pathol. 1999 Oct;23(10):1233-40.

Rickinson AB, Kieff E (2006) Epstein-Barr Virus. In: Knipe DM, Howley PM, editors. Fields Virology. 5 ed. Philadelphia: Lippincott Williams & Wilkins. pp. 2655–2700.

Rochford R, Cannon MJ, Moormann AM. Endemic Burkitt's lymphoma: a polymicrobial disease? Nat Rev Microbiol. 2005 Feb;3(2):182-7.

Rowe M, Lear AL, Croom-Carter D, Davies AH, Rickinson AB. Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. J Virol. 1992 Jan;66(1):122-31.

Sáez AI, Artiga MJ, Sánchez-Beato M, Sánchez-Verde L, García JF, Camacho FI, Franco R, Piris MA. Analysis of octamer-binding transcription factors Oct2 and Oct1 and their coactivator BOB.1/OBF.1 in lymphomas. Mod Pathol. 2002 Mar;15(3):211-20.

Saha A, Robertson ES. Epstein_Barr Virus-Associated B-cell Lymphomas: Pathogenesis and Clinical Outcome. Clin Cancer Res. 2011 May 15;17(10):3056-3063.

Saridakis V, Sheng Y, Sarkari F, Holowaty MN, Shire K, Nguyen T, Zhang RG, Liao J, Lee W, Edwards AM, Arrowsmith CH, Frappier L. Structure of the p53 binding domain of HAUSP/USP7 bound to Epstein-Barr nuclear antigen 1 implications for EBV-mediated immortalization. Mol Cell. 2005 Apr 1;18(1):25-36.

Santón A, Martín C, Manzanal AI, Preciado MV, Bellas C. Paediatric Hodgkin's disease in Spain: association with Epstein-Barr virus strains carrying latent membrane protein-1 oncogene deletions and high frequency of dual infections. Br J Haematol. 1998 Oct;103(1):129-36.

Schmitz R, Hansmann ML, Bohle V, Martin-Subero JI, Hartmann S, Mechtersheimer G, Klapper W, Vater I, Giefing M, Gesk S, Stanelle J, Siebert R, Küppers R. TNFAIP3 (A20) is a tumor suppressor gene in Hodgkin lymphoma and primary mediastinal B cell lymphoma. J Exp Med. 2009;206(5):981–989.

Seitz V, Hummel M, Marafioti T, Anagnostopoulos I, Assaf C, Stein H. Detection of clonal T-cell receptor gamma-chain gene rearrangements in Reed-Sternberg cells of classic Hodgkin disease. Blood. 2000 May 15;95(10):3020-4.

Skinnider BF, Mak TW. The role of cytokines in classical Hodgkin lymphoma. Blood. 2002 Jun 15;99(12):4283-97.

Sleckman BG, Mauch PM, Ambinder RF, Mann R, Pinkus GS, Kadin ME, Sherburne B, Perez-Atayde A, Thior I, Mueller N. Epstein-Barr virus in Hodgkin's disease: correlation of risk factors and disease characteristics with molecular evidence of viral infection. Cancer Epidemiol Biomarkers Prev. 1998;7:1117-21.

Smuk G, Illés A, Keresztes K, Kereskai L, Márton B, Nagy Z, Lacza A, Pajor L.Pathol Oncol Res. Pheno- and genotypic features of Epstein-Barr virus associated B-cell lymphoproliferations in peripheral T-cell lymphomas. 2010 Sep;16(3):377-83.

Sorg UR, Morse TM, Patton WN, Hock BD, Angus HB, Robinson BA, Colls BM, Hart DN. Hodgkin's cells express CD83, a dendritic cell lineage associated antigen. Pathology. 1997 Aug;29(3):294-9.

Staratschek-Jox A, Kotkowski S, Belge G, Rüdiger T, Bullerdiek J, Diehl V, Wolf J. Detection of Epstein-Barr virus in Hodgkin-Reed-Sternberg cells : no evidence for the persistence of integrated viral fragments in Latent membrane protein-1 (LMP-1)-negative classical Hodgkin's disease. Am J Pathol. 2000;156:209-16.

Steidl C, Telenius A, Shah SP, Farinha P, Barclay L, Boyle M, Connors JM, Horsman DE, Gascoyne RD. Genome-wide copy number analysis of Hodgkin Reed-Sternberg cells identifies recurrent imbalances with correlations to treatment outcome. Blood. 2010 Jul 22;116(3):418-27.

Steidl C, Connors JM, Gascoyne RD. Molecular pathogenesis of Hodgkin's lymphoma: increasing evidence of the importance of the microenvironment. J Clin Oncol. 2011 May 10;29(14):1812-26.

Straathof KC, Leen AM, Buza EL, Taylor G, Huls MH, Heslop HE, Rooney CM, Bollard CM. Characterization of latent membrane protein 2 specificity in CTL lines from patients with EBV-positive nasopharyngeal carcinoma and lymphoma. J Immunol. 2005 Sep 15;175(6):4137-47. Erratum in: J Immunol. 2005 Nov 1;175(9):6238.

Szczepański T, Beishuizen A, Pongers-Willemse MJ, Hählen K, Van Wering ER, Wijkhuijs AJ, Tibbe GJ, De Bruijn MA, Van Dongen JJ. Cross-lineage T cell receptor gene rearrangements occur in more than ninety percent of childhood precursor-B acute lymphoblastic leukemias: alternative PCR targets for detection of minimal residual disease. Leukemia. 1999 Feb;13(2):196-205.

Sugden, B. 1989. An intricate route to immortality. Cell 57:5–7.

Swerdlow, S.H., Campo, E., Harris, N.L., Jaffe, E.S., Pileri, S.A., Stein, H., Thiele, J., Vardiman, J.W. Swerdlow et al Classification of Tumours of Haematopoietic and Lymphoid Tissues, Fourth Edition. Lyon, France:IARC Press; 2008

Tanijiri T, Shimizu T, Uehira K, Yokoi T, Amuro H, Sugimoto H, Torii Y, Tajima K, Ito T, Amakawa R, Fukuhara S. Hodgkin's reed-sternberg cell line (KM-H2) promotes a bidirectional differentiation of CD4+CD25+Foxp3+ T cells and CD4+ cytotoxic T lymphocytes from CD4+ naive T cells. J Leukoc Biol. 2007 Sep;82(3):576-84.

Taub R, Kirsch I, Morton C, Lenoir G, Swan D, Tronick S, Aaronson S, Leder P.Thorley-Lawson DA, Gross A. Persistence of the Epstein-Barr virus and the origins of associated lymphomas.N Engl J Med. 2004 Mar 25;350(13):1328-37. Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci U S A. 1982 Dec;79(24):7837-41.

Tinguely M, Rosenquist R, Sundström C, Amini RM, Küppers R, Hansmann ML, Bräuninger A. Analysis of a clonally related mantle cell and Hodgkin lymphoma indicates Epstein-Barr virus infection of a Hodgkin/Reed-Sternberg cell precursor in a germinal center. Am J Surg Pathol. 2003 Nov;27(11):1483-8.

Trivedi P, Zhang QJ, Chen F, Minarovits J, Ekman M, Biberfeld P, Klein G, Winberg G. Parallel existence of Epstein-Barr virus (EBV) positive and negative cells in a sporadic case of Burkitt lymphoma. Oncogene. 1995 Aug 3;11(3):505-10.

Tzankov A, Zimpfer A, Pehrs AC, et al. Expression of B-cell markers in classical Hodgkin lymphoma: a tissue microarray analysis of 330 cases.. Mod Pathol 2003:16:1141-1147.

Tzankov A, Bourgau C, Kaiser A, Zimpfer A, Maurer R, Pileri SA, Went P, Dirnhofer S. Rare expression of T-cell markers in classical Hodgkin's lymphoma. Mod Pathol. 2005 Dec;18(12):1542-9.

Uchida J, Yasui T, Takaoka-Shichijo Y, et al. Mimicry of CD40 signals by Epstein-Barr virus LMP1 in B lymphocyte responses. Science 1999;286:300–303.

van Dongen JJ, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003: 17:2257-2317.

Vasto S, Candore G, Balistreri CR, Caruso M, Colonna-Romano G, Grimaldi MP, Listi F, Nuzzo D, Lio D, Caruso C. Inflammatory networks in ageing, age-related diseases and longevity. Mech Ageing Dev. 2007 Jan;128(1):83-91.

Vockerodt M, Morgan SL, Kuo M, Wei W, Chukwuma MB, Arrand JR, Kube D, Gordon J, Young LS, Woodman CB, Murray PG. The Epstein-Barr virus oncoprotein, latent membrane protein-1, reprograms germinal centre B cells towards a Hodgkin's Reed-Sternberg-like phenotype. J Pathol. 2008 Sep;216(1):83-92.

Wang D, Liebowitz D, Kieff E. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. Cell. 1985 Dec;43(3 Pt 2):831-40.

Watanabe K, Yamashita Y, Nakayama A, et al. Varied B-cell immunophenotypes of Hodgkin/Reed-Sternberg cells in classic Hodgkin's disease. Histopathology 2000: 36:353-361.

Weber-Matthiesen K, Deerberg J, Poetsch M, Grote W, Schlegelberger B. Numerical chromosome aberrations are present within the CD30+ Hodgkin and Reed-Sternberg cells in 100% of analyzed cases of Hodgkin's disease. Blood. 1995;86(4):1464–1468.

Weiss LM, Strickler JG, Warnke RA, Purtilo DT, Sklar J. Epstein-Barr viral DNA in tissues of Hodgkin's disease.Am J Pathol. 1987 Oct;129(1):86-91.

Weiss LM, Movahed LA, Warnke RA, Sklar J. Detection of Epstein-Barr viral genomes in Reed-Sternberg cells of Hodgkin's disease. N Engl J Med. 1989 Feb 23;320(8):502-6.

Weiss L M. EBV-Associated Lymphoproliferative Disorders: Update and Clinical Significance of New Entities (2012); From:http://www.uscap.org/site~/100th/pdf/companion05h01.pdf

Weniger MA, Melzner I, Menz CK, Wegener S, Bucur AJ, Dorsch K, Mattfeldt T, Barth TF, Möller P. Mutations of the tumor suppressor gene SOCS-1 in classical Hodgkin lymphoma are frequent and associated with nuclear phospho-STAT5 accumulation. Oncogene. 2006;25(18):2679–2684.

White RE, Groves IJ, Turro E, Yee J, Kremmer E, Allday MJ. Extensive co-operation between the Epstein-Barr virus EBNA3 proteins in the manipulation of host gene expression and epigenetic chromatin modification. PLoS One. 2010 Nov 15;5(11).

White RE, Rämer PC, Naresh KN, Meixlsperger S, Pinaud L, Rooney C, Savoldo B, Coutinho R, Bödör C, Gribben J, Ibrahim HA, Bower M, Nourse JP, Gandhi MK, Middeldorp J, Cader FZ, Murray P, Münz C, Allday MJ. EBNA3B-deficient EBV promotes B cell lymphomagenesis in humanized mice and is found in human tumors. J Clin Invest. 2012 Apr 2;122(4):1487-502.

Wlodarska I, Nooyen P, Maes B, Martin-Subero JI, Siebert R, Pauwels P, De Wolf-Peeters C, Hagemeijer A. Frequent occurrence of BCL6 rearrangements in nodular lymphocyte predominance Hodgkin lymphoma but not in classical Hodgkin lymphoma. Blood. 2003 Jan 15;101(2):706-10.

Yates J, Warren N, Reisman D, Sugden B. A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc Natl Acad Sci U S A. 1984 Jun;81(12):3806-10.

Zettl A, Lee SS, Rüdiger T, Starostik P, Marino M, Kirchner T, Ott M, Müller-Hermelink HK, Ott G. Epstein-Barr virus-associated B-cell lymphoproliferative disorders in angloimmunoblastic T-cell lymphoma and peripheral T-cell lymphoma, unspecified. Am J Clin Pathol. 2002 Mar;117(3):368-79.

Zheng B, Fiumara P, Li YV, Georgakis G, Snell V, Younes M, Vauthey JN, Carbone A, Younes A. MEK/ERK pathway is aberrantly active in Hodgkin disease: a signaling pathway shared by CD30, CD40, and RANK that regulates cell proliferation and survival. Blood. 2003;102(3):1019–1027.

Zhimin He, Baozhong Xin, Xinhai Yang, Ching-ping Chan and Liang Cao. Nuclear Factor-κB Activation Is Involved in LMP1-mediated Transformation and Tumorigenesis of Rat-1 Fibroblasts.Cancer Res 60, 1845–1848, April 1, 2000

Zhou XG, Sandvej K, Li PJ, et al. Epstein-Barr virus (EBV) in Chinese pediatric Hodgkin disease: Hodgkin disease in young children is an EBV-related lymphoma. Cancer 2001:92:1621-1631.