Addis Ababa University,

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Hodgkin Lymphoma at a Tertiary cancer center in Ethiopia: novel biomarkers and characterization of the tumor microenvironment in relation to Epstein Barr Virus (EBV)

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Hodgkin Lymphoma in Ethiopia: novel biomarkers and characterization of the tumor microenvironment in relation to Epstein Barr Virus (EBV)

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Dedication

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List of Abbreviations

ABVD	Adriamycin, bleomycin, vinblastine, dacarbazine
ALCL	Anaplastic large B-cell lymphoma
APRIL	A proliferation-inducing ligand
ASCT	Autologous stem cell transplantation
BCL6	B-cell lymphoma 6, a transcription repressor
BEACOPP	Bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisolone
BL	Burkitt lymphoma
BOB1	POU domain class 2-associating factor 1, transcription coactivator
BV	Brentuximab-Vedotin
CAR-Tcell	Chimeric antigen receptor
CBC	Complete blood cell count
CD	Cluster of differentiation
CHL	Classical Hodgkin lymphoma
CI	Confidence interval
CMAF	Belong to leucine zipper transcription factors
c-Met	Mesenchymal-epithelial transition factor, receptor tyrosine kinase
COPDAC	Cyclophosphamide, vincristine sulfate, prednisone, and dacarbazine
CT scan	Computed tomography scan
CXCL10	Chemokine ligand 10
CXCL9	Chemokine ligand 9
DAB	3,3`diaminobenzidine

DLBCL	Diffuse large B-cell lymphoma
EBER	Epstein Barr Viruss encoded-RNA
EBNA	Epstein-Barr virus nuclear antigens
EBV	Epstein Barr Virus
EFS	Event free survival
ESR	Erythrocyte sedimentation rate
FFPE	Formalin fixed paraffin embedded tissue
FISH	Fluorescent *in situ* hybridization
FoxP3	Forkhead box P3
GLOBOCAN	Global Cancer Observatory
H&E	Hematoxylin and eosin
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDCT	High dose chemotherapy
HIV	Human immune deficiency virus
HL	Hodgkin lymphoma
HR	Hazard ratio
HRP	Horseradish peroxide
HRS	Hodgkin and Reed-Sternberg
IFNg	Interferon gama
IFRT	Involved field radiotherapy
IHC	Immunohistochemistry
IMP3	Insulin-like growth factor II messenger RNA-binding protein 3

INCTR	International Network for Cancer Treatment and Research
IPS	International Prognostic Score
L&H	Lymphocytic and histiocytic
LP	Lymphocyte predominant
LAG3	Lymphocyte activation gene3
LDCHL	Lymphocyte depleted classical Hodgkin lymphoma
LMP1	Latent membrane protein 1
LRCHL	Lymphocyte rich classical Hodgkin lymphoma
MCCHL	Mixed cellularity classical Hodgkin lymphoma
MHC	Major histocompatibility
ML	Malignant lymphomas
MST1R	Macrophage stimulating 1 receptor
MUM1	Multiple myeloma 1/interferon regulatory factor 4
NHL	non-Hodgkin lymphoma
NLPHL	Nodular lymphocyte predominant Hodgkin lymphoma
NSCHL	Nodular sclerosis classical Hodgkin lymphoma
2-Oct	Organic cation transporter 2
OS	overall survival
PAX5	Paired box5 transcription factor, B-cell specific activator protein
PBS	Phosphate buffered saline
PD1	Programmed cell death protein 1
PDL1	programmed death ligand 1
PET	Positron emission tomography

TASH	Tikur Anbessa Specialized Hospital
T-bet	T-box transcription factor
Th 2	T-helper 2
Th1	T-helper 1
THRLBCL	T-cell/histocyte-rich large B-cell lymphoma
TMA	Tissue microarray
TME	Tumor microenvironment
TNFAIP2	Tumor necrosis factor A inducible protein 2
T-reg	regulatory T-cell

Abstract

Classification and identification of Hodgkin lymphoma (HL) relies on morphological and biomarker-based diagnosis. However, CD30 and CD15 used for the identification of HL, are not expressed on all tumor cells. This gap can be narrowed by combining detection of the insulin-like growth factor II messenger RNA-binding protein 3 (IMP3). This study aimed to confirm the diagnostic value of IMP3. In addition to investigating the prevalence of EBV among HL cases, the tissue cellular composition of EBV-related and EBV-unrelated cases also was assessed. Furthermore, the treatment outcomes and prognostic impact of FoxP3 and PD1 in the tumor microenvironment (TME) of classical HL (CHL) were determined. Clinical records of consecutive patients with HL diagnosed between the years 2014 and 2019 were reviewed. A tissue microarray (TMA) of 126 of CHL and nodular lymphocyte predominant HL from Tikur Anbessa Hospital was constructed. TMA sections were usedfor immunohistochemical staining and detection of Epstein Barr Virus encoded-RNA. The stained immune cells were quantified by HALO 2.3. A total of 91(68.4%) patients were male and the male-to-female ratio was 2.2:1 with a median age of 22 years. The majority of the cases, 67 (50%) were of the mixed-cellularity and 40 (40%) of the nodular-sclerosis subtype. A total of 77 (61.1%) of HL cases expressed LMP1/EBER. The immunoreactivity of HL cases to IMP3 was determined and compared with the commonly used biomarkers for HL diagnosis. 122 (96.8%), 95 (75.4%), and 126 (100%) of the cases were positive for CD30, CD15, and IMP3 markers, respectively. Infiltration of CD8+, T-bet+, and FoxP3+ cells was higher in the TME of EBV-related CHL, with *P* values of <0.001, <0.001 and <0.016, respectively. In contrast, PD1 expression was higher in the TME of EBV-unrelated CHL ($P < 0.001$). In a Kaplan-Meir analysis, the 9-year overall survival (OS) and event free survival (EFS) was 78.6% and 66.5%, respectively. The patients in

the high-risk group, International Prognostic Score (IPS ≥ 3), had inferior OS and EFS compared to the patients in the low-risk group (IPS< 3) (*P*= 0.04 for both survival outcomes). HIV-associated HL had inferior EFS (*P*= 0.016). Patients with low lymphocyte (≤ $0.6x10^9$ cells/L) had inferior OS (HR= 10.9; 95% CI, 1.4-84; *P*= 0.016) and EFS (HR= 5.9; 95% CI, 1.7-20; *P*= 0.005). Patients with high FoxP3+ cells (≥ 9%) and high PD1+ cells (≥ 24.6%) in the TME of CHL had poor prognosis. The 9-year OS for high FoxP3 was 64.6% with (HR= 4.3; 95% CI, 1.2-15.7; *P*= 0.02) and the 9-year OS for high PD1 was 57.1% with (HR=3.4; 95% CI, 1.2-15.7; *P*=0.03). Low lymphocyte count, high FoxP3+, and high PD1+ were all significantly associated with adverse overall survival in a multivariate Cox-regression. The present study supports previous reports that suggest the potential of using IMP3 as a diagnostic biomarker for HL. The study findings further highlight the previously unrecognized possibility that distinct immunosuppressive mechanisms involving FoxP3+ and PDI-expressing cells may be in play within EBV-positive and negative HL types. Although the treatment outcomes is relatively inferior compared to high-income countries, a significant proportion of patients with CHL, are cured when provided adequate treatment. Overall, HIV-associated CHL, low lymphocyte count, high FoxP3, and PD1 were associated with unfavorable treatment outcomes.

Keywords: Histopathological Patterns of HL, Insulin-like growth factor II messenger RNA-binding protein 3, Epstein Barr Virus, Tumor Microenvironment, Treatment outcome, FoxP3, PD1, Prognosis

1. Introduction

Cancer affects all human beings worldwide. However, it becomes an even more complex and challenging disease in the transitioning countries where the population is also challenged by many types of life-threatening infectious diseases. Moreover, the health systems in such countries are poorly prepared to deal with such challenges (Gopal et al., 2012). According to the Global Cancer Observatory (GLOBOCAN) report, the new cancer cases and deaths worldwide were estimated to be 19.3 and 10 million, respectively. Overall, the report showed that the burden of cancer has shifted to the transitioning countries (Sung et al., 2021). In Africa, cancer has become a public health problem and a major cause of death due to socioeconomic and cultural factors (Bahnassy et al., 2020). The share of cancer deaths in Africa (7.2%) is higher than its share of incidence (5.7%). Whereas the share of cancer incidence in Europe (22.8%) and the USA (20.9%), their shares of cancer deaths account for 19.6% and 14.2%, respectively (Sung et al., 2021). In Africa, it is estimated that cancer incidence and death might increase up to 70% by 2030 (Parkin et al., 2014). In addition, cancer in Africa has been coexisting with communicable diseases like AIDS, tuberculosis, malaria, and the most recent pandemic COVID-19 (Halperin, 2020, Gutman et al., 2020). In Sub-Saharan Africa, a notably high cancer incidence, with over a million new cases and 800,000 cancer related deaths are expected for 2030 (Gopal *et al*., 2012). By comparison to cancer, the mortality rate of HIV, tuberculosis, and malaria across sub-Saharan Africa, is estimated to be 460,000 (UNAIDS, 2021), 377, 000 (WHO, 2020), and 596.488 in 2019 (Max and Hannah, 2019) respectively. In Ethiopia, cancer is becoming one of the major public health problems, with an estimated 60,000 new cases diagnosed each year (Tigeneh et al., 2015).

1.1. Overview of Lymphoma

Malignant lymphomas (ML) are lymphoid neoplasms, with high incidence both in high and low-income countries (Baris and Zahm, 2000, Omoti and Halim, 2005). MLs include various types of lymphoid neoplasms, such as Hodgkin's lymphoma (HL), and non-Hodgkin lymphoma (NHL) (Mwakigonja et al., 2010), with high morbidity and mortality rates in sub-Saharan Africa (Kaaya et al., 2006, Tomoka et al., 2018). HL was the first lymphoid malignancy to be described by Thomas Hodgkin in 1832 in a paper titled "On some morbid appearances of the absorbent glands and spleen" (Hodgkin, 1832). HL is a distinct form of lymphoma, with specific clinical characteristics. Although the causes of HL are not known definitely (Küppers et al., 2002), some infections such as Epstein-Barr virus (EBV), immune deficiencies, inherited factors, age, sex, and geographical location, all contribute to the risk (Saarinen et al., 2013).

1.2. Hodgkin Lymphoma

1.2.1. Hodgkin lymphoma prevalence

Hodgkin Lymphoma is a distinctive lymphoid neoplasm with a characteristic clinical presentation, epidemiology, and histopathological pattern (Mwakigonja et al., 2010). HL affects all age groups (children, adolescents, adults, and the elderly). However, it was reported to be most common in age groups between 20 to 34 years (Shanbhag and Ambinder, 2018). Overall, lymphomas represent 21% of cancer in adolescents aged 14-19 years and almost two-third of which are HL, constituting 11% of all cancers in childhood (1-14 years). Further, of all lymphomas and reticuloendothelial malignancies, one-quarter was reported as HL (Siegel et al., 2017). In high-income countries, HL shows a bimodal distribution with about fifty percent of HL diagnosed between ages 15 and 35 years, and a second incidence peak which was observed after the age of fifty years (Thomas et al., 2002). In low-income

countries, HL is among the most common cancers in adolescents and younger adults (Olu-Eddo and Omoti, 2011). However, reports from these countries have shown that HL is more commonly diagnosed in children than in early adulthood, and the second peak does not occur (Riyat, 1992, Tefera et al., 2016).

The global incidence of HL is about 3 in 100,000 per year (Torre et al., 2015). According to the GLOBOCAN 2018 report, the 5-year prevalence for HL in the world, Africa, and Ethiopia was 275,947 (3.6%), 25,721 (2.0%) and 1660 (1.5%), respectively. Similarly, the estimated mortality rate in 2018 was 26,167 (0.30%), 4,229 (0.48%), and 352 (0.40%) in the World, Africa and Ethiopia, respectively (IRAC, 2020).

Studies conducted to assess the pattern of cancer in Ethiopia have shown a considerable number of patients diagnosed with HL (Tefera et al., 2016). Recent estimates indicate that hematological malignancies comprise the third leading cause of cancer and cancer related mortality in Ethiopia, of which nearly 20% are HL (Woldeamanuel et al., 2013). A study conducted in Northwest Ethiopia showed that lymphoma accounted for 17.2% (93/540) of cancer patients diagnosed between 2014 and 2015. Out of those 20% were HL and 27% of the total lymphoma cases diagnosed were children of age <14 years old (Tefera et al., 2016).

1.2.2. Hodgkin lymphoma classification and cellular origin

HL has a unique histo-morphological characteristic with a small proportion of neoplastic cells, which comprise <1% of the total cell population, and a majority of non-malignant immune cells (Aldinucci et al., 2010). HL is divided into two types - classical Hodgkin lymphoma (Brousset et al., 1991), which accounts for 90% of all cases, and the less common nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) form. The CHL is sub-typed into

nodular sclerosis (NSCHL), lymphocyte rich (LRCHL), mixed cellularity (MCCHL), and lymphocyte depleted (LDCHL) subtypes (Swerdlow et al., 2016), depending on the morphology, types, and abundance of infiltrating cells, phenotypic, genotypic, and clinical findings (Harris et al., 1994). In high and middle-income countries, the predominant subtype is NSCHL (Belkaid et al., 1995, Leoncini L et al., 1996), while in low-income countries MCCHL and LDCHL subtypes are predominant (Riyat, 1992, Kusuda et al., 1998).

HL is characterized by the presence of Hodgkin and Reed-Sternberg HRS cells in classical Hodgkin lymphoma (CHL) and lymphocytic and histiocytic (L&H) or lymphocyte predominant (LP) cells in nodular lymphocyte predominant HL (NLPHL) (Bräuninger et al., 2006). These cells are large, abnormal, mononucleated Hodgkin cells and multinucleated giant tumor cells that are derived from B lymphocytes. The presence of these cells, along with other cells in the background, helps pathologists diagnose HL. The vast majority of infiltrating cells around the tumor are T lymphocytes, macrophages, histiocytes, fibroblasts, eosinophilic granulocytes, bystander B lymphocytes, and plasma cells (Englund et al., 2016).

Recently the NLPHL has been categorized into nodular lymphocyte predominant B-cell lymphoma by the International Consensus Classification ICC of lympho-haematopoietic neoplasm, while the 5th edition of the WHO classification of haematolymphoid tumors (WHO-HAEM5) has maintained the term NLPHL (Alaggio et al., 2022, Falini et al., 2022). The cellular origin of HRS cells in CHL remained enigmatic for a long period of time due to their unusual immunophenotype, which is not related to any type of the normal hematopoietic cells (Küppers et al., 2002). Although their B-cell origin is now established, based on the presence of monoclonal immunoglobulin rearrangement and expression of B-lineage markers such as CD19, CD20, CD22, sIg, or CD79a, the latter markers either not present, or can only be found

in a small proportion of HRS cells (Thomas et al., 2002). In contrast, they express markers of other cell types, including CD30 (a member of TNF-growth factor), a receptor of cytokine (Wendtner et al., 1995), CD15 (granulocytes), Perforin (T-cells) (Thomas et al., 2002) (< 10% cases), and lack of CD45 (common leukocyte antigen) (Stein et al., 2017). The tumor cells of NLPHL, LP, differ from the tumor cells of CHL, HRS, in that they have a preserved B-cell program and consistently express B-lineage markers such as PAX5, CD19, CD20, CD22, CD79a, sIg, as well as OCT2 and its coactivator BOB1(von Wasielewski et al., 1997b).

1.2.3. Viral etiology of Hodgkin lymphoma

Several studies have pointed to the viral etiology of HL and suggested Epstein-Barr virus (EBV) as a transforming agent in HL (Gutensohn, 1982). The EBV was the first virus to be linked to human cancer and was isolated from Ugandan children with jaw tumors 50 years ago by Denis Burkitt together with Michael Epstein and Yvonne Barr (Gopal et al., 2012). Subsequently, EBV has been associated with several types of cancer, including Burkitt lymphoma, HL, B-cell NHL, and nasopharyngeal carcinoma, and EBV has been recognized for its ability to transform B-lymphocytes (Thorley-Lawson and Gross, 2004, Fitzmaurice et al., 2017). EBV acquisition differs in high-income countries from that in Sub-Saharan Africa.

Sero-prevalence of EBV is 75% in developed countries, whereas its prevalence is estimated to be 80-100% throughout sub-Saharan African countries with acquisition later in life coincident with sexual activity and in early childhood through contact with oral secretions in high-income and low incomes countries, respectively (Biggar et al., 1978, Cohen, 2000, Gopal et al., 2012). Even though EBV infects the majority of the world's population, there are regional differences in the incidence of EBV-associated cancers. The variations in incidence highlight the complex

interplay between host genetics, environmental factors, and viral genetic variation. Notably, EBV is associated with BL and HL in sub-Saharan Africa (Chang et al., 2009). It is important to continue researching and understanding these factors to better comprehend the unique association of EBV with BL and HL in Sub-Saharan Africa. In HL, the incidence of EBV is different depending on the geographical location and histopathologic variants. The virus is detected in 30% to 50% of patients in the USA, Brazil, Europe (Glaser et al., 1997), Taiwan (Chang et al., 2008), and the United Arab Emirates (Al-Salam et al., 2008), whereas it infects nearly 100% of the children with HL in the developing countries (Thomas et al., 2002, Dinand et al., 2007, Castillo et al., 2011).

EBV may play a significant role in the development of certain types of lymphoma. The EBV-encoded RNA (EBER-1) has been detected in >50% of lymphomas from sub-Saharan Africa, the highest rate of association is seen in BL and HL (Mwakigonja et al., 2010). Similarly, a study comparing the epidemiological variation of EBV in HL for Kenyan and Japanese patients showed that EBV-encoded RNA was found in 79% and 59% of the Kenyan and Japanese cases, respectively (Kusuda et al., 1998). In addition, HIV may create a potential oncogenic synergy among co-infected HL patients by increasing B-lymphocytes infected with EBV while decreasing the cytotoxic activity of T-cells against EBV-infected B cells (Mwakigonja et al., 2010).

EBV is a humanγ-1 herpes virus that has a tropism for B-lymphocytes, where it can establish latent infection. The genome of EBV is a DS-DNA molecule with 170-kilobase that encodes >85 genes (Thompson and Kurzrock, 2004). EBV expresses a limited set of genes during latent infection including three integral proteins known as the latent membrane proteins (LMP-1, -2A, and -2B), two Epstein-Barr virus encoding RNA (EBER-1 and -2) and six Epstein-Barr virus

nuclear antigens (EBNA-1, EBNA-2, -3A, -3B and -3C, and the lead protein EBNA-LP) (Chang et al., 2009).

EBV genome has been detected in the malignant cells of HL cases. However, there is variation in the prevalence of EBV and the highly associated subtype of HL with EBV between low- and high-income countries. Studies have demonstrated that EBV is associated with the MCCHL subtype than with the NSCHL subtype (Glaser et al., 1997), but a study from Kenya showed a strong association of EBV with both MCCHL and NSCHL (Leoncini L et al., 1996). HL in children, elderly, men, and cases living in disadvantaged social conditions is highly associated with EBV with HL (Caporaso et al., 2009). In general, the frequency of EBV in HL cases is higher in low-income countries. However, underprivileged patients and children from high and middle-income countries show a similar frequency of EBV positivity in HL (Zarate-Osorno et al., 1995). In low-income countries, 92% of HL cases harbor the DNA of EBV (Leoncini L et al., 1996), while in western countries 15-30% of NSCHL and 70% of MCCHL have been associated with EBV (Brousset et al., 1991).

The human immune deficiency virus is considered a risk factor for many types of cancer in developing and resource-limited countries. A study from the USA reported that around 3.8% of HL cases were associated with HIV at the time of diagnosis (Shiels et al., 2014). The incidence of HL has increased in sub-Saharan Africa during the burden of HIV (Casper, 2011). Probably, HIV increases the risk of HL in resource-limited countries due to loss of immunity (Bohlius et al., 2011). The immune-suppressive status might lead to the activation of EBV on B-lymphocyte precursors, which contributes to the alterations of some antigen-activated B cells in the germinal center into transformed tumor cells (Guiguet et al., 2009). Furthermore,

EBV has been detected in HRS tumor cells of HL cases with HIV (Carbone et al., 2017). Most HL cases with HIV are of MCCHL and LDCHL subtypes (Grewal et al., 2018).

1.2.4. Hodgkin lymphoma cellular microenvironment

HRS cells of HL are interspersed among a heterogeneous population of nonmalignant reactive cells. The immune cells in the tumor microenvironment (TME) of HL are recruited by the abnormal network of cytokines and chemokines. The transformed HRS cells express receptors that attracted rosetting T-cells (Carbone et al., 1995), eosinophils and mast cells with CD30 ligands, and neutrophils with proliferation-inducing ligand (APRIL) (Küppers, 2009). Due to crosstalk between the tumor cells and the stromal cells in the TME of HL, the HRS cells received survival signals from the immune cells in the TME, and the HRS cells produce signals inducing differentiation and proliferation of T-cells, macrophages, and fibroblasts (Aldinucci et al., 2010).

Recently, several studies focussed on the tumor microenvironment (TME), due to its potential role in the development and progression of various cancers, including HL. Understanding the TME of HL has important prognostic and therapeutic implications as it can influence the behaviors of cancer cells and their response to treatments (Moskowitz et al., 2014, Ansell et al., 2015). The HL microenvironment in the tumor tissue is rich with reactive inflammatory cells, including the T-cells (Steidl et al., 2011). However, the immune cells present in the tumor do not impose an effective anti-tumor immune response. In contrast, these immune cells might support the growth and proliferation of HRS cells (Ansell, 2015). However, the abundance and role of T-helper cells have continued to be a controversial topic in the microenvironment of HL. Some studies have suggested a Th-1 mediated response to be

predominant in the HL microenvironment (Marshall et al., 2004, Steidl et al., 2011). Greaves *et al*. (2013) showed that the predominant T-lymphocyte in the microenvironment of HRS cells is of Th-1subtype, being activation marker-rich, cytokine-secretory, and proliferative rather than being Th-2 and T-reg. A recent study indicated that the majority of cytotoxic CD8+ T-cells in EBV+ CHL are present due to a Th-1 response as Th-1 cells stimulate a cellular immune response (Wu et al., 2016). In contrast, other studies have suggested a Th-2 polarization (Poppema and van den Berg, 2000, Schreck et al., 2009). The regulatory T-cells (Treg) have also been detected in CHL (Marshall et al., 2004). The accumulation of Th2 cells and T-reg in the TME of HRS provides a protective environment for HRS cells (Poppema and van den Berg, 2000, Marshall et al., 2004). This may explain how HRS cells escape anti-tumor immunity.

EBV in CHL might be involved in the genetic dysfunction of tumor cells; LMP1 constitutively activates several signaling cascades and pathways in tumor cells (Kieser and Sterz, 2015, Liao et al., 2020), which can induce the production of cytokines and chemokines such as CXCL9 and CXCL10 used for the recruitment of various types of immune cells (Kis et al., 2006, Chetaille et al., 2009). However, the immune response is suppressed in TME of EBV-related CHL due to the presence of regulatory T-cells and impaired production of LAG3, IFNγ (Gandhi et al., 2006). In addition, EBV-infected tumor cells evade T-cell responses through the modulation of antigen presentation pathway, as the virus downregulates the expression of major histocompatibility I and II (MHC class I and II) on the tumor cells (Reichel et al., 2015).

Programmed cell death 1 (PD1) belongs to the immunoglobulin super family which is involved in the immune checkpoint to regulate immune responses in order to prevent host tissue damage (Wherry et al., 2007). Tumor-infiltrating lymphocytes express the programmed death

ligand PD1 (Ahmadzadeh et al., 2009), and the HRS cells in CHL express the programmed death ligand 1 (PDL1) as an immune escape mechanism (Yamamoto et al., 2008). T cells expressing programmed cell death protein1 (PD1) are immune-suppressed through interaction with its ligands PD-L1/2 (Nishimura et al., 1999). However, studies on the PD1 expression in the TME of CHL are scarce and conflicting (Yamamoto et al., 2008, Carbone et al., 2009, Muenst et al., 2009), although there is evidence that HRS malignant cells of HL express the PD1 ligand (PD-L1) (Steidl et al., 2010, Roemer et al., 2016). A recent report showed that high expression of PD1 and PDL1 in the TME of CHL was associated with inferior treatment outcome (Hollander et al., 2017).

Regulatory T-cells (T-reg), FoxP3+ cells, play an important role in the regulation of the immune system in the host to prevent autoimmunity and overactivation of immune response in a given infection (Malek and Bayer, 2004, Askenasy et al., 2008). The suppressive potential of T-reg is due to the expression of the Forkhead box protein P3 (FoxP3) (Lu et al., 2017). The regulator T-cells are present in the TME of various types of tumors, high density of T-reg in the TME has been related to either poor or good prognosis in malignancies (Kelley and Parker, 2010). The T-cells in the TME in CHL are of Th2 and T-reg subtypes, this may explain the immune impairment and persistence of tumor cells (Vardhana and Younes, 2016). In CHL, the cellular infiltrate plays a role in suppressing the cytotoxic immune responses (Aldinucci et al., 2010).

The transcription factor FoxP3 belongs to the forkhead helix family. FoxP3 regulates gene expression in T-reg cells, and it is critical for the identity and function of T-reg cells (Lu et al., 2017). In HL, T-reg cells are known to accumulate in the tumor microenvironment, which can

inhibit the antitumor immune response. Several studies found that high expression of FoxP3 in T-reg was associated with a poor response to chemotherapy and worse survival outcomes in HL(Alvaro et al., 2005, Asano et al., 2006, Kelley et al., 2007). Despite these findings, the prognostic value of FoxP3 expression in HL remains controversial (Chetaille et al., 2009, Koreishi et al., 2010). Therefore, further research is necessary to determine the role of FoxP3 in HL prognosis and to identify potential therapeutic targets.

1.3. Immunohistochemistry (IHC) and HL diagnosis

1.3.1. Diagnostic markers currently used for HL

Immunohistochemical phenotyping has contributed new insights into the understanding of the nature, the classification, and the diagnosis of Hodgkin lymphoma. It has been considered an ideal tool for evaluating Hodgkin Reed-Sternberg cells (Ferry and Harris, 1996). The tumor HRS cells of CHL commonly express the diagnostic markers CD30 and CD15 and rarely express CD20 and BCL6 whereas the tumor (LP) cells of nodular lymphocyte predominant Hodgkin lymphoma express CD20, CD40, BCL6, OCT2, and BOB1 (Küppers et al., 2002). Especially CD15 serves as a diagnostic biomarker for giant cells that do not always show the typical Reed-Sternberg morphology (Von Wasielewski et al., 1997a). A combination of morphological and immunohistochemical tests are used for the classification and differential diagnosis of HL. According to the World Health Organization guideline (Swerdlow et al., 2016) the immunophenotypes of the malignant cells in classical Hodgkin lymphoma and nodular lymphocyte predominant Hodgkin lymphoma differ significantly.

Hodgkin and Reed-Stenberg cells of CHL are stained positive for CD30 and CD15; they are occasionally positive for CD20 and negative for CD45. In contrast, the malignant cells (LP) of NLPHL consistently express the B-cells biomarkers CD20 and CD45, but they lack CD30 and

CD15 (Eichenauer et al., 2014). Since some of the immune markers that are used for the diagnosis of HL also are expressed on the tumor cells of large cell lymphomas, the differential diagnosis of HL from diffuse large B-cell lymphoma (DLBCL), anaplastic large B-cell lymphoma (ALCL), and the T-cell/histocyte-rich large B-cell lymphoma (THRLBCL) is more complicated (Stein et al., 2017). In addition, some HRS cells lack the typical immunohistochemistry (IHC) characteristics (Karnik et al., 2003). Hence, evaluation and validation of new immune markers for HL diagnosis is of paramount importance.

1.3.2. Potential new markers for HL diagnosis

Since some of the malignant cells of HL, the HRS and the LP cells, lack the typical immunohistochemistry (IHC) characteristics, several new IHC markers have been reported for the diagnosis of CHL and NLPHL. These include Insulin-like growth factor II and messenger RNA-binding protein 3 (IMP3). IMP3 is expressed in embryos, but down regulated later in adult tissues. IMP3 is apparently expressed in several types of tumors including the HRS and LP of HL. Tang and co-workers, detected IMP3 in almost all cases of CHL, which is superior to the IHC of CD15, CD30, PAX5, and MUM1 (Tang et al., 2013). Additional IHC biomarkers have been reported for the diagnosis of CHL, such as TNFAIP2- the tumor necrosis factor A inducible protein 2 (Kondratiev et al., 2011), CD137 (Anderson et al., 2012), c-MET- Mesenchymal-epithelial transition factor (Xu et al., 2012), CD163 (Bedewy et al., 2013), and MST1R-macrophage stimulating 1 receptor (Wha Koh et al., 2013). However, some of these biomarkers showed controversial results (Zhang and Aguilera, 2014). In contrast, IMP3 seems to be a promising immunohistochemical biomarker for diagnosing Hodgkin lymphoma, particularly in cases where the Hodgkin Reed-Stenberg HRS cells exhibit weak or absent expression of the diagnostic CD30 and CD15 markers (Zhang and Aguilera, 2014). IMP3 may

provide additional diagnostic value in such challenging cases, aiding accurate identification of HL. In addition, IMP3 is expressed in (80-100%) of Burkitt lymphoma (BL), a diffuse large B-cell lymphoma (DLBL), and follicular lymphoma (King et al., 2009).

1.4. Treatment and prognosis in HL

HL is a treatable and often curable form of cancer, especially when diagnosed early. Advances in treatment such as chemotherapy, radiation therapy, targeted therapy, and immunotherapy have significantly improved the prognosis of HL. However, a proper clinical evaluation is required to categorize HL patients into different stages to follow an appropriate therapeutic strategy and staging is an important tool for risk stratification (Hasenclever et al., 1998). The Ann Arbor staging system in lymphoma focus on the sites of tumor (nodal and extra nodal) location, and the absence or presence of systemic symptoms (Rosenberg, 1977). For accurate staging, physical examination, and detailed history with special emphasis on the presence or absence of B-symptoms are required (Ansell, 2016). The common systemic symptoms in HL include weight loss, fever, night sweats, and other symptoms such as fatigue and pruritus. The presence of B-symptoms influences both prognosis and treatment choices in HL (Gallamini et al., 2016). In addition, laboratory studies such as complete blood cell count (CBC), blood chemistry tests, erythrocyte sedimentation rate (ESR) and screening for viral infection such as HIV, HBV and HCV will improve prognostic accuracy.

The staging is according to the anatomical site and number of lymph nodes and involvement of extra-nodal sites (Hingorjo and Syed, 2008). Further, the four stages are classified into A and B based on the presence or absence of B-symptoms. These staging and classifications of the HL cases are performed at the time of disease diagnosis and prior to placing the patient

in a definitive therapy (Gallamini et al., 2016). In addition, staging of HL requires a chest x-ray, contrast enhanced computed tomography scan (CT) of the abdomen, neck, and chest (Cheson et al., 2014). Positron emission tomography (PET) has been recommended for staging of HL and assessment of treatment response (Barrington et al., 2014). However, bone marrow biopsy is carried out for staging in resource-limited countries (El-Galaly et al., 2012, Eichenauer et al., 2014). The international prognostic scores IPS, including age>45 and male sex, physical examination, laboratory tests (such as serum albumin <4 g/dl, hemoglobin <10.5 g/L, white blood cells count ≥15x10^9/L and lymphocyte count <0.6x109/L) are currently used for determination of prognosis in advanced stage CHL (Hasenclever et al., 1998).

The general practice for treating HL is based on classifying patients into non-bulky stage I, and stage II, which are early stages; and stage III and IV that are advanced stages. Also, stage IIB and stage II with bulky disease are commonly considered as advanced stages (Cheson et al., 2014). Currently, HL adult patients are treated with chemotherapy by using adriamycin, bleomycin, vinblastine, and dacarbazine (ABVD) (Canellos and Niedzwiecki, 2002), followed by involved field radiotherapy (IFRT) based on their stage (Eichenauer et al., 2014). Advanced-stage patients (in both adults and children) are treated with Bleomycin, Etoposide, Doxorubicin, Cyclophosphamide, Vincristine, Procarbazine, Prednisolone (BEACOPP) (Kelly et al., 2011). The treatment of childhood HL is based on risk groups, which are divided according to the prognostic factors used for the classification and staging of adulthood HL. Low and intermediate-risk childhood HL are generally treated with a combination of radiation and chemotherapy. Whereas high-risk childhood HL is treated with a higher dose combination of radiation and chemotherapy (Mauz-Körholz et al., 2010, Mauz-Körholz et al., 2015, Daw et al., 2020). Cyclophosphamide, vincristine sulfate, prednisone, and dacarbazine COPDAC can

be used in the treatment regimen of intermediate and advanced-stage pediatric HL cases (Mauz-Körholz et al., 2010).

Most patients diagnosed with HL can be cured if placed on standard treatment protocol according to their risk group. In children, the treatment failure rates are 15% and 20% for patients in the low and advanced stages, respectively (Daw et al., 2020), The cure rate in adults is up to 80% in advanced stage, and >90% in early stage (Moccia et al., 2012). 15-20% of HL patients develop resistance to therapy or relapse after treatment, and the prognosis of patients with relapse is still poor (Evens et al., 2012). Relapse/refractory HL patients are treated with high-dose chemotherapy (HDCT) with autologous stem cell transplantation (ASCT) (Moskowitz et al., 2001).

The use of prognostic factors related to tumor genetics and tumor microenvironment, tailored to the therapy response might be of great value (Cuccaro et al., 2014). Immunotherapy based treatments are used currently as a third line therapy for relapsed and refractory HL, such as targeted with Brentuximab-Vedotin (BV) (Senter and Sievers, 2012, Moskowitz et al., 2015). PD1 inhibitors are clinically approved in 2016 to be used in the treatment of relapse/refractory HL cases (Younes et al., 2016). Immuno-checkpoint inhibitors open a new era in the treatment of HL. However, the treatment outcome and response to PD1 inhibitor vary and uncertain (Armand et al., 2016, Xu-Monette et al., 2018). In addition, evidence indicates toxicity of these agents (Johnson et al., 2022). A recent ongoing clinical trial has also shown the use of chimeric antigen receptor (CAR T-cell) therapy to treat HL (Ramos et al., 2020).

In general, the treatment outcome for HL patients is favourable, except for the patients at high risk. To date, the prognostication of HL depends on clinical factors which include, history, clinical finding, CBC, ESR, albumin level and other laboratory values. Accurate prognostication of HL outcome is crucial in determining the appropriate treatment choice, and it allows to tailor therapy to the specific need of the patients, considering factors such as the presence of specific protein in the cancer cells or other prognostic indicators (Rathore and Kadin, 2010, Xie et al., 2023).

1.5. The rationale of the study

Ethiopia is one of the sub-Saharan Africa countries in which cancer is becoming one of the major public health problems (Solomon and Mulugeta, 2019) . Tikur Anbessa Specialized Hospital (Yamamoto et al.) is the first cancer treatment center in the country. Previously, this center was the only facility for cancer patients (including HL) seeking diagnosis and treatment. Recently, a few cancer treatment centers have been inaugurated at different regions in Ethiopia (Alemu and Hailemariam, 2022). Unfortunately, not all patients diagnosed with cancer can afford the cost of travelling to the centers and treatment. As a result, most cancer patients have no choice but stay in their villages and try alternative traditional medicine (Tuasha et al., 2018). In addition, the absence of a cancer registry throughout the country except for the recently launched one by the Addis Ababa city administration, contributes to the low estimation of cancer incidence and mortality rate in the country. Among the patients that have the opportunity to receive diagnosis and treatment in TASH, 10% of them had early stage and 90% were found to have advanced or unknown stage (Tigeneh et al., 2015).

In sub-Saharan Africa, the hematologic malignancies, non-Hodgkin lymphoma (NHL), leukemia, Hodgkin lymphoma (HL) are the major causes of morbidity and mortality (Gopal et al., 2012). According to the few studies conducted on the pattern of all cancers, HL is diagnosed commonly among Ethiopian patients especially during childhood at Tikur Anbessa and Gondar referral hospitals (Woldeamanuel et al., 2013, Tefera et al., 2016). HL is one of the common cancer diseases at young adult ages, and considered as a curable disease in patients from high-income countries (Flowers and Armitage, 2010). In contrast, as reported by the GLOBOCAN the mortality rate is high in Ethiopia (IRAC, 2020). This might be due to the low public health priority given in Ethiopia to cancers in general and HL in particular as a result of limited resources and other pressing infectious diseases, such as HIV, malaria and tuberculosis. Thus, it is very difficult to estimate the real picture of epidemiology, incidence, and mortality rate of HL in Ethiopia. This study is designed to elucidate age distribution, clinical characteristics, histopathological subtypes, and survival outcome of HL in an Ethiopian setting.

Despite an increasing burden of HL in children and adults in Ethiopia, no specialized diagnostic methods are available. The absence of techniques such as IHC, cytogenetic, molecular, and fluorescent *in situ* hybridization (FISH), which are the core diagnostic technologies in hematopathology, in high- and middle-income countries, contribute to the low accuracy of diagnosis and sub-typing. Many HL biomarkers (such as CD30, CD15, CD20, CD138, BLC6, OCT2, BOB1, CD3, CD40, CD79a, PD1, PDL1 and PDL2) have been recognized as diagnostic and therapeutic targets (Swerdlow et al., 2016, Younes et al., 2016). However, some HRS and LP cells of HL do not express these diagnostic biomarkers. Therefore, in this study, the diagnostic capacity of novel biomarkers will be assessed and verified by IHC.

Several novel biomarkers have been proposed to be used for HL diagnosis (Kondratiev et al., 2011, Anderson et al., 2012, Bedewy et al., 2013, Wha Koh et al., 2013). Among these, the IMP3 protein has been reported to be expressed in various malignancies, including lung cancer (Xu et al., 2007), pancreatic cancer (Lok et al., 2014), colon cancer (Li et al., 2009), gastric cancer (Damasceno et al., 2014), liver cancer (Jeng et al., 2008), and renal cancer (Jiang et al., 2006). The overexpression of IMP3 protein has been linked to adverse outcomes in cancers of the bladder, lung, stomach, pancreas, and the adenocarcinoma of the esophagus (Burdelski et al., 2018). Yet, a contradicting report is available regarding the prognostic value of IMP3 protein in solid tumors (Chen et al., 2017). Furthermore, several studies have suggested the diagnostic value of IMP3 for HL, particularly in cases with weak or absent expression of HL diagnostic markers (Zhang and Aguilera, 2014, Tang et al., 2013). Thus, this study was designed to also evaluate the diagnostic and prognostic values of IMP3 expression on HL patients, treated at a tertiary cancer treatment center in Ethiopia.

Many studies have suggested an association of EBV in the pathogenesis of HL (Carbone et al., 2017). However, its relation to EBV shows a wide geographic variation in its epidemiology (Glaser et al., 1997). Therefore, this study will assess virological etiology of HL in Ethiopia. Furthermore, the role of EBV infection in the recruitment of immune cells in the tumor microenvironment of HL will be evaluated, and the prognostic values of the cells will be assessed. Since the prevalence of EBV with HL is different between high-income and resource-limited countries (Brousset et al., 1991, Leoncini L et al., 1996), this study aimed to determine the cellular components of the microenvironment of EBV-related and EBV-unrelated HL using IHC in the patients from Ethiopia.

1.6. Significance of the study

Since this study estimates the burden, the population at risk and treatment outcome of HL and the association of EBV with HL, it will serve as a landmark description of HL in Ethiopia. The identification of immunophenotypic characteristics of HL may provide novel biological markers that can be used for diagnosis by using IHC. In addition, the study will identify possible prognostic markers and treatment targets for HL. The identification of such markers could be a basis for the physicians to follow the status of the patients and help them choose appropriate management approaches. The findings of this study will add to the existing knowledge on HL and may deepen the knowledge of the role of EBV in the pathogenesis of HL.

1.7. Hypothesis

The frequency of association of EBV infection with HL in Ethiopia is different from that reported from the Western countries, and the immune cells in the TME could be used for prognostication and prediction of the disease outcome.

1.8. The Research questions

1. What are the demographic, histopathologic patterns, and EBV status of Hodgkin lymphoma in Ethiopia?
2. Does the international prognostic score predict treatment outcomes in HL patients in Ethiopia?
3. Does EBV infection determine the cellular components of the HL tumor microenvironment?

4. Could the composition of the HL tumor microenvironment predict treatment outcomes?
5. Could IMP3 serve as a useful additional diagnostic marker in HL?

1.9. Objectives of the study

1.9.1 General objective

The general objective of this study was to assess the prevalence and role of EBV in the recruitment of immune cells in the tumor microenvironment of HL and to evaluate diagnostic and prognostic immune markers that predict the treatment outcomes in HL patients.

1.9.2 Specific objectives

1. To assess the demographic and histopathologic patterns of HL in Ethiopia
2. To evaluate treatment outcomes of HL in Ethiopia in relation to the international prognostic factors
3. To investigate the association of HL with EBV infection in Ethiopia
4. To investigate whether EBV infection determines the composition of cellular tumor microenvironment of CHL
5. To Evaluate the diagnostic power of the biomarker IMP3 in HL
6. To evaluate the prognostic value of the immune cell composition in the tumor microenvironment of HL

2. Material and Methods

2.1. Study setting and design

This study was a single center hospital-based study. For this study, formalin fixed paraffin embedded tissue (FFPE) blocks of HL patients diagnosed between the year 2014 and 2019 were collected from the archive of the Department of Pathology, Tikur Anbessa Specialized Hospital, TASH, Addis Ababa, Ethiopia. Sections from whole tissue and tissue microarray (TMA) were prepared for subsequent procedures and analysis. Data on demographics, risk factors, symptoms, disease stage, laboratory parameters, HIV status, type of treatment and treatment cycles, and follow-up data were collected from the clinical records of the HL patients from the hematology clinic of TASH. Laboratory work and analysis were conducted at the biomedical science laboratory of the Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, Pathology Department of Armauer Hansen Research Institute (AHRI), and immunohistochemistry laboratory, Department of Oncology, Lund University, Sweden.

2.2. Description of study site

Tikur Anbessa Specialized hospital, TASH, is the main medical education facility and tertiary academic hub providing multidisciplinary services in Ethiopia. The hospital is the main referral facility that serves the entire population of Ethiopia. It receives a large number of patients including critically sick cases. Previously, all cancer patients from all around the country were referred to this hospital. TASH is treating only about 1% of these patients. 500 adults and children with hematologic malignancy were seen in the hospital's oncology unit every year. The pediatric oncology unit has been established in 2012 by the International

Network for Cancer Treatment and Research (INCTR) at TASH. The common cancers seen in children at TASH are leukemia, lymphoma, retinoblastoma, Wilm's tumor and bone and soft tissue sarcomas (Memirie et al., 2018).

2.3. Sample size and sampling technique

2.3.1. Sample size

The sample size was calculated using the 5-year cases of HL in Ethiopia that has been reported by the GLOBOCAN 2012 which was 1815 (Ferlay et al., 2015), And the proportion of EBV infection on HL in Africa. Accordingly, the sample size for this study was 138 with confidence level of 95% and level of significance of 5%.

The sample size was determined by using the following formula:

$$n_0 = \frac{Z^2 pq}{e^2}$$

Where n_0 is the desired sample size, Z is the standard normal deviation (1.96), P is the proportion of the EBV on HL cases in Africa (Thomas et al., 2002), which is estimated to be (0.9), q=1-p, and e is degree of accuracy desired (0.05).

$$n_0 = \frac{1.96^2\, x0.9x(1-0.9)}{0.05^2}$$

$$n_0 = 138$$

Where n_0 is the sample size without considering the finite population correction factor (fpc). Applying the fpc factor results in the actual sample size *n.* The target population with

Hodgkin's lymphoma was (N = 1815), so the required actual sample size (n) was estimated by using the following formula:

$$n = \frac{n_0 N}{n_0 + (N - 1)}$$

Where n is the desired sample size applying the fpc, n_0 is the sample size without considering the fpc and N is the estimate of the target population size (Naing et al., 2006). Accordingly, the actual sample size (n) for EBV prevalence on HL cases was 129. Finally, we choose the higher sample size which was 138.

2.3.2. Study population

The target population of this study was all HL patients in Ethiopia. The source population was HL patients who visited TASH between the year 2014-2019. The study population was HL patients who visited the hematology and pediatric oncology clinic at TASH between the years 2014 and 2019 and those who had FFPE tissue blocks at the pathology department of TASH and fulfilled the inclusion criteria.

2.3.3. Sampling technique

HL patients were recruited for this study based on the availability of Formalin Fixed Paraffin Embedded (FFPE) tissue blocks at the Department of Pathology, TASH. Since follow-up data is required for the evaluation of the prognostic factors and treatment outcomes, HL patients who had available clinical records and FFPE tissue blocks were included in the study.

2.3.4. Inclusion criteria

- Adults and parents or legal guardians of children who were willing to participate in the study and were able to sign written consent.
- All age groups (including children <5 years)
- Ann Arbor stage I-IV
- Diagnosis of HL at the pathology laboratory of TASH
- Tissue blocks preserved at the pathology laboratory of TASH

2.3.5. Exclusion criteria

Blocks with small tissue size

2.4. Ethical clearance

Ethical clearance was obtained from Addis Ababa University College of Natural Sciences and College of Health Sciences institutional ethics review boards, AHRI institutional ethical review board and the national ethical review committee. Informed written consent was collected from patients and parents or legal guardians of children included in the study in accordance with the declaration of Helsinki. Upholding safety, confidentiality, and privacy of the patients and materials were ensured. FFPE tissue blocks were processed both in Ethiopia and Sweden, therefore, a material transfer agreement was signed between the collaborating institutions in the study from Ethiopia and Sweden.

2.5. Patient recruitment and sample collection procedures

The principal investigator had a meeting prior to the recruitment with the health professionals (clinicians, pathologist, and nurses) involved in recruitment of patients and data

collection. During the meeting the study protocol, the inclusion and exclusion criteria were discussed. Using the eligibility criteria, the investigator/study team determined suitable candidates for the protocol. The candidates for this study were patients attending to the hematology clinic at TASH either as new cases or referred from other health facilities throughout the country and diagnosed with Hodgkin lymphoma between the year 2014 and 2019. The eligible candidates or parents/legal guardians/ of the cases were clearly informed about the study and its importance. Those who agreed to participate in the study signed the consent form prior to enrolment. Information on the patient's history, physical examination and staging were done and collected by the physician and was introduced into the patients' clinical records. Data on laboratory investigations, treatment, risk factors and demographic characteristics were collected according to the form attached in the appendix. Routinely, biopsy from enlarged lymph nodes are fixed and used to diagnose HL at TASH. Therefore, this study used the FFPE tissue blocks prepared and archived in the Department of Pathology, TASH. Tissue blocks with adequate preserved sample were included in the downstream analysis. Retrieval of tissue blocks, and clinical data were carried out according to data safety procedures.

2.6. Preparation of formalin fixed paraffin embedded (FFPE) tissue blocks

FFPE block sections were prepared in the Department of Pathology, TASH for routine diagnosis purposes. Briefly, tissue samples were cut into thin slices using a sterilized scalpel immediately after excision. The sliced tissue was placed inside histology cassettes before processing. The cassettes with corresponding samples were stored inside sealed containers full of fixatives (10% formalin). The fixative volume was 15 to 20 times higher than the specimen volume. Then tissue samples were infiltrated in wax as follows; the tissue was

dehydrated on 3 alcohol baths with growing concentrations of 70%-85%-90%, water was removed by 3 final absolute alcohol baths, cleared on 3 toluene baths, and then the wax was infiltrated in the tissue by using hot wax baths (44°C - 60 °C). Finally, the tissue was embedded inside a mold filled with hot paraffin. Tissue standard operating procedures (Canene-Adams, 2013) have been followed during the preparation of FFPE.

2.7. Tissue Micro Array construction (TMA)

For the construction of TMA, representative areas on the tissue were selected from H&E and CD30 stained sections by a hematopathologist (AK), and the area was marked on the slide using a microscope. Then, the region of interest was marked on the corresponding tissue blocks, and 4 regions of 1mm^2 size were selected (multiple cores from each sample). Small punches (0.6 mm) were removed from donor tissue blocks and placed into the recipient block in specific row/column orientation (Figure SI) (Kononen et al., 1998, Hedvat et al., 2002, Tzankov et al., 2003). Information about the columns and rows was maintained on a TMA map. The TMA was constructed using a manual tissue arrayer (Pathology Devices, Inc, USA). Sections were prepared from TMA blocks and mounted on slides for further studies. TMA sections were used for standard H&E, immunohistochemistry staining, and *in situ* hybridization.

2.8. Hematoxylin and Eosin (H&E) staining

Tissue sections (whole section and tissue microarray (TMA) sections) were mounted on slides, dried overnight, fixed using incubator at 60^{0}C for 1 hr, and stained for microscopic examination by hematoxylin and eosin (H&E). The H&E staining procedure of tissue sections was performed as follow; tissue sections were fixed at 60^{0}C for 1 hr. and cooled at room

temperature, then the sections were de-paraffinized: on 2 changes of xylene for 5 minutes, then moved to jars with 99% and 96% ethanol, and finally washed in a jar with distilled water (sections were immersed for 5 minutes at each step). Then sections were stained with Mayer's hematoxylin for 10 minutes, then rinsed in tap water and counter-stained with eosin for 3 minutes. Then, sections were dehydrated with distilled water for 30 seconds followed by ethanol 96% for 30 seconds; ethanol 99% for 5 minutes, and 2 times xylene for 5 minutes each. H&E control (positive and negative) slides were stained before each batch of new test slides for quality control. Previously diagnosed HL cases at the pathology department of TASH, after staining, the slides were examined by a hematopathologist and consultant hematopathologist. Then blinded testing of the cases was conducted for re-classifying HL subtypes by two hematopathologists. As recommended in previous studies (Iyengar, 2009), discordant results were resolved by consensus.

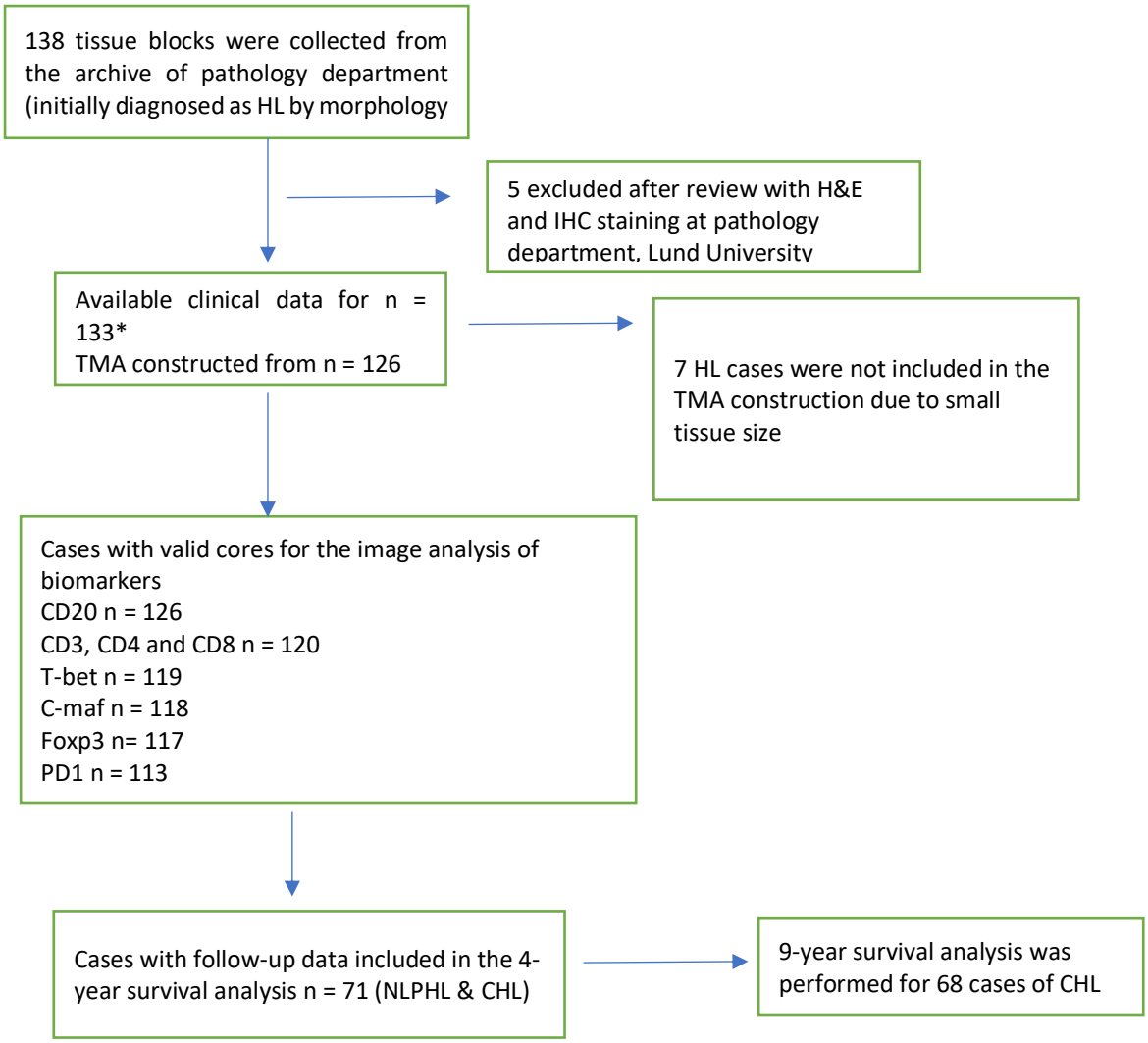


Figure 1. Flow chart of patients included in the study. HL patients diagnosed between 2014 and 2019 at TASH.

* 7 patients with small tissue materials were not included in the construction of TMA.

2.9.Immunohistochemistry

2.9.1. Tissue section preparation

Whole tissue and TMA sections (3-4mm) thickness were prepared by using a manual rotary microtome. Sections mounted on poly-L-lysine-coated slides were air dried overnight, and fixed on the oven at 60^0C for 1hr. The slides were left at room temperature for cooling and then after either stained or stored at 4^0 C.

2.9.2. Antigen retrieval for IHC

The dried, fixed, cooled tissues were antigen retrieved before immunohistochemistry staining either by using PT-link for pretreatment (DAKO plus) without deparaffinization or in microwave oven (900W) after deparaffinization (Von Wasielewski et al., 1994). The tissue sections were antigen retrieved either in high pH or in low pH (Kim et al., 2004) according to the monoclonal antibody requirement. The following was the pretreatment procedure using microwave oven in detail. That is, tissue sections were de-paraffinized through two changes of xylene, for 5 minutes each. Sections were hydrated by dipping them in absolute ethanol (99.5%), 96% ethanol and distilled water for 5 minutes each. Then, slides with sections were placed in microwave slide dish (plastic works best with a plastic slide rack) and covered with citrate buffer for pH 6 or EDTA solution or other buffer used for pretreatment for pH 9. The microwave was set on high power (800W) until boiling, for about 8 minutes. Then, the effect was reduced to 300 W for 15 minutes (time may vary by microwave, but the important thing is to make sure the slides boil rapidly for 10 minutes minimum and do not dry out. An additional container of citrate buffer may be microwaved together with the slides to allow for refilling of the slide dishes.). the slides were left for a minimum of 20-30 minutes for cooling, rinsed in tap water for 5 minutes and then placed in wash buffer phosphate-buffered saline (PBS) for 5 minutes. Excess PBS was dried from slides and tissue was circled with hydrophobic pen leaving 1mm space between tissue and circle.

2.9.3. Immunohistochemistry (IHC) staining

As previously described (Cordell et al., 1984), the antigen-retrieved tissue sections were stained by using DAKO/Agilent Envision™ Flex reagent for IHC; Endogenous peroxidase was inactivated, primary antibodies were added, Horse radish peroxidase enzyme attached to the

secondary antibody by dextran backbone (EnVision™) from DAKO was used for the indirect visualization of the primary antibody bound to the specific cell marker and for visualization 3,3`diaminobenzidine (DAB) was used. DAKO/Agilent Envision reagents were used for IHC. Tissue sections were stained either manually or using DAKO-autostainer-plus with the following procedure: endogenous peroxidase activity was blocked using by incubating the tissue section with 3% H_2O_2 solution from DAKO kit for 5 minutes. Sections were rinsed with phosphate buffered saline (PBS) twice and incubated with primary antibody or negative control solution for 30 minutes. Then rinsed with PBS twice and incubated with EnVision horseradish peroxide (HRP-conjugate polymers) DAKO kit for 30 minutes. Sections were rinsed with PBS twice and incubated with 3,3`diaminobenzidine (DAB) for 10 minutes. Then after sections were rinsed with distilled water twice and counterstained with hematoxylin for 4 minutes. Finally, sections were rinsed with tap water 3 times, dehydrated using 95%-99% ethanol-3 changes of xylene for 5 minutes each and mounted by protex mounting medium.

2.10. Clinical, histopathological and immunophenotypic data acquisition from HL patients

The clinical records of 133 HL cases were reviewed from which demographic data, histopathological subtype of HL, stage of the disease and other related clinical data were extracted. At TASH, staging of patients with HL include; detailed history with special attention to the presence or absence and duration of systemic (B) symptoms and pruritus; adequate surgical biopsy; physical examination with particular emphasis to lymph node regions and organs; complete blood count and differential, ESR; plain chest-x-rays with measurement of mediastinal mass; abdominal ultrasonographic studies; CT scan of the neck, chest, abdomen, and pelvis; and bone marrow aspiration and/or biopsy for stage IV disease, and stage III if the

patient had cytopenias. Since there is no electronic cancer or death registry system, patients whose records lacked follow-up data, were contacted through their cellphone numbers which were available on the clinical records. Accordingly, 71 cases of NLPHL & CHL cases were included for the 4-year overall survival analysis, and 9-years survival analysis was performed for 68 cases of CHL.

Two whole sections with 3-4 µm thickness were prepared from each tissue blocks and stained with hematoxylin and eosin (H&E) and CD30. The stained sections were used for classification of HL into CHL and NLPHL subtypes. Cases were reviewed by a specialist in hematopathology and reassessed according to 2016 WHO classification of Tumors of Hematopoietic and Lymphoid Tissues (Swerdlow et al., 2016).

Slides with TMA sections were stained with the following monoclonal antibodies (m-Abs); CD30, CD15, PAX5, CD20, CD79a, CD45, OCT-2, PD-1, CD57, CD3 and LMP-1 to characterize and sub-classify HL. PD-1 and CD57 markers were used for the differential diagnosis of NLPHL, CHL and T-cell histocyte rich large B-cell lymphoma (THRLBCL) (Stein H, 2017). Polymer-based IHC techniques were used to stain the TMA and tissue sections with the Abs (Cordell et al., 1984). The primary antibodies details and dilutions shown in Table 1. Similarly, IHC technique was applied for staining the TMA tissue sections with IMP3 antibody, to detect the validity of using IMP3 as a supplementary biomarker for HL diagnosis.

2.11. Description of the biomarkers used to determine the immune cells in the microenvironment of HL

To study the type and proportion of the immune cells in the microenvironment of HL and to compare the cellular component of HL between EBV-related and EBV-unrelated cases, we

used a panel of immunomarkers. The TMA sections were prepared and stained with the biomarkers (monoclonal Antibodies) using IHC as described previously.

The biomarkers used for this study were monoclonal antibodies (m-Ab) against CD20 (B-cell marker), CD3 (T-cell marker), CD4 (non-cytotoxic T-cell marker), CD8 (cytotoxic T-cell marker), FOXP3 (T_{reg} cell marker), T-bet (Th-1 marker), CMAF (Th-2 marker), PD1(marker for exhausted effector cells), and PDL1 (the program cell death ligand). To determine the predominant T-cell types in the microenvironment of HL, the immune-stained cells were quantified using HALO digital pathology image analysis software and the proportion of each cell in the microenvironment of HL cells were estimated. The proportion of cells were related to HL subtypes, EBV-expression, age, and sex, as well as to patient outcome to evaluate the prognostic value of the immune cells of the HL microenvironment. The m-Ab dilutions and details are presented in Table 1.

2.12. Quality control assurance and Quantification of IHC

To optimize IHC for our study, before using the newly arrived m-Ab in the studied samples, positive and negative controls were used to test the quality of the m-Abs. For quality control, four cores of 1mm^2 were created for each sample, and two cores of reactive tonsils were added at each batch of TMA (as described previously). The tissue material (whole sections and TMA sections) was assessed by two researchers and one hematopathologist with consultation from a senior hematopathologist. Valid cores with high number of tumor cells were selected for quantification, areas with fibrosis and cases with poor quality cores were excluded. The automated quantification of positive immune cells was performed by HALO 2.3, a platform for image analysis from Indica Lab Inc©.

2.13. Quantification of IHC stained immune cells in the microenvironment of HL

The images of the stained TMA were prepared using high quality Philips image scanning technique at a magnification of x20 for HALO platform analysis. The scanned images were loaded in a computer with HALO software at the pathology department at Skane University Hospital in Malmo, Sweden. The cytonuclear v1.6 algorithm was used to count the membranous and nuclear cell biomarkers, the software was trained to differentiate between the membrane and nuclear staining of the cells in the TME, and adjusted for the cellular parameters and annotation of the area to be scored (Koelzer et al., 2019). The threshold for each core was set based on the pixel´s value. The quantified data by HALO software was extracted in CSV format containing raw data for each core, then converted into XL format. The extracted data contains data on total cells/mm^2, biomarker positive cells/mm^2, percentage of biomarker positive cells/mm^2, average cell area/µm^2, average cytoplasmic area/µm^2 and average nuclear area/µm2 of the quantified cells/mm2, for each core.

Table 1. Characteristics of the primary antibody used in the immunohistochemistry tests

Antigen	Clone	Dilution	Supplier	Antigen-retrieval method
CD30	Ber-H2	1:50	Agilent/DAKO	PT-Link pH9
CD15	Carb-3	1:50	Agilent/DAKO	PT-Link pH9
PAX5	SP34	1:200	CellMarque	PT-Link pH9
CD20cy	L26	1:500	Agilent/DAKO	PT-Link pH9
CD3	A0452	1:200	Agilent/DAKO	PT-Link pH9
CD79a	JCB117	1:500	Agilent/DAKO	PT-Link pH9
CD45	2B11+PD7/26	1:300	Agilent/DAKO	PT-Link pH9
CD57	TB01	1:100	Agilent/DAKO	PT-Link pH9
OCT-2	EPR16570	1:1000	Abcam	PT-Link pH9

PD-1	NAT105	1:100	CellMarque	2100 Retriever pH6
PDL1	22C3	1:50	Agilent/DAKO	2100 Retriever PH6
IMP3	69.1	1:100	Agilent/DAKO	PT-Link pH9
LMP1	CS1-4	1:100	CellMarque	PT-Link pH9
CD4	4B12	1:60	Agilent/DAKO	PT-Link pH9
CD8	C8/1244B	1:100	Agilent/DAKO	PT-Link pH9
FOXP3	236A/E7	1:200	Abcam	PT-Link pH9
T-bet	EPR9302	1:500	Abcam	PT-Link pH9
C-maf	EPR16484	1:200	Abcam	PT-Link pH9

2.14. *In situ* hybridization for EBER-1

In situ hybridization for EBV early RNA was carried out according to a protocol previously described (Lee et al., 2013). EBER-ISH was performed using the Ventana BenchMark XT automatic immunostainer following the manufacturer's recommendations (Ventana Medical Systems, Tucson, AZ, USA), Figure 2. A digoxigenin-labeled (DIG) oligonucleotides complementary to portions of the EBV-encoded early RNA transcripts (EBER probe cat.no 800-2842), and (negative control cat.no 800-2847) were used according to the manufacturer's protocol. Briefly, the deparaffinized FFPE tissue sections were pretreated with cell conditioning solution 2 (CC2) at pH 6 for 24 minutes at 86^0C and the proteins were digested with protease 3 enzyme for 28 minutes. The sections were then rinsed with saline sodium citrate (SSC) buffer. Genomic DNA and the EBER-probe were denatured at 80^0C for 12 minutes followed by hybridization at 55^0C for 60 minutes. Stringency wash was performed using sodium citrate, sodium chloride (SSC 10×) to wash off unbound or weakly bound probes.

Hybridization of the probe to the targeted nucleic acid sequence was visualized using ISH iView Blue Detection Kit.The labelled probe was located by an anti-DIG antibody and a biotin-

conjugated secondary antibody, which were incubated for 20 and 8 minutes, respectively. This step was followed by the addition of a Streptavidin-AP (alkaline phosphatase) enzyme conjugate which binds to the biotin present on the secondary antibody and MgCl2 solution as an enhancer for 16 and 4 minutes, respectively. The complex was then visualized with 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium (NBT) chromogen which was applied for 32 minutes. All reactions were conducted at 36^0C. The sections were counterstained by red stain II for 4 minutes, dehydrated, and mounted. Positivity was defined as a clear blue precipitate labeling in the nuclear area of the HRS cells, which were readily detected by light microscopy. For the quality control, positive and negative controls were included in each batch.

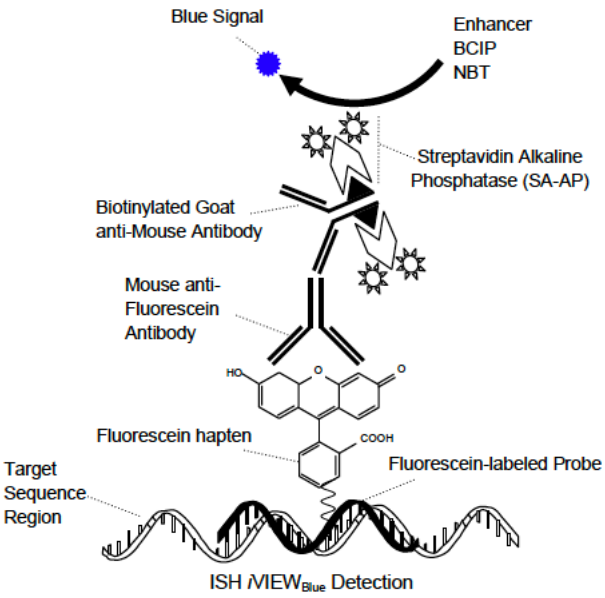


Figure 2. *ISHiVIEW* Blue Plus Reaction for Fluorescein-labelled Probe (www.ventana.com, 2012)

2.15. **Statistical analysis**

For statistical analysis, SPSS version 28.0.0.0 (190) was used, and the normal distribution of continuous data was checked by the Shapiro-Wilk test.

2.14.1 Statistical analysis for clinical and histopathological characteristics

Descriptive statistics were used to explore the demographic characteristics, clinical variables, and treatment modalities. Chi-square (x^2) test or Fisher's exact test was used to show the differences and distributions of different variables. Mann-Whitney U and Kruskal-Wallis tests were used to analyze the distribution of age among nominal and categorical variables, respectively. Analysis of variance or univariate analysis was used to assess mean differences of age among the groups of categorical variables and mean differences of immune cells in the microenvironment.

2.14.2 Qualitative and quantitative measurement of CD30 and IMP3

CD30 and IMP3 expression on the HRS cells were evaluated qualitatively by scoring the intensity of the membranous staining as (weak, moderate, and strong). Chi-square test or Fisher's exact test was used to analyse the association of IMP3 expression on HRS cells with the clinicopathological features of HL. CD30 and IMP3 stained and unstained HL tumor cells were counted for all cases in mm^2 core tissue using the high-power magnification (x40) by a pathologist, then the percentage of HL tumor cells expressing CD30 and IMP3 proteins was calculated. The mean difference between the percentage of CD30 and IMP3 protein expression on HRS cells was analysed by using Wilcoxon Signed Ranks test. The differences in the expression of IMP3 and CD30 between groups of continuous and categorical data were compared using two tailed T-test, Mann-Whitney U and Kruskal-Wallis tests as appropriate. A result with *P value* less than 0.05 was considered statistically significant.

2.14.3 Association of EBV antigens expression with clinicopathological features

The x^2 and Fisher exact test were used to analyze the association of LMP1 and EBER expression with the binary and categorical variables related to the clinical data. To test the independent association of clinical characteristics of CHL with EBV marker expression, the data was analyzed by the logistic regression model.

2.14.4 The proportion of immune cells in the microenvironment of EBV-related and EBV-unrelated HL

Descriptive statistics were conducted to analyze the mean, standard deviation, and minimum and maximum count of the immune cells in the microenvironment of HL. Wilcoxon means rank test or paired sample T-test was performed to compare the proportion of immune cells. Correlations between different biomarkers were determined using Spearman's correlation test. The Mann-Whitney test or independent sample T test was performed as appropriate to determine the association of biomarkers expression with the demographic and clinical features of HL. The proportion of immune cells in the microenvironment of EBV-related and EBV-unrelated CHL cases was compared using the Mann–Whitney test or independent sample T-test as appropriate for variables with binary outcomes, and the Kruskal–Wallis test or one-way ANOVA was conducted for categorical variables. CHL cases with HIV infection and NLPHL cases were excluded from the comparison analysis of the biomarkers in TME of EBV-related and EBV-unrelated CHL. To determine the factors that were independently associated with the high expression of a given biomarker linear regression analysis was conducted. All outcome variables with a *P*-value of ≤ 0.25 were entered in the linear regression model.

2.15. Treatment and follow-up data

All 133 patients in this study were initially treated with the standard chemotherapy regimen doxorubicin, bleomycin, vinblastine, and dacarbazine (ABVD). Nine, 6.8% of the patients, all belonging to the pediatric population, were treated with ABVD followed by cyclophosphamide, vincristine sulfate, prednisone, and dacarbazine (COPDAC). Five (3.8% of the patients), received ABVD and COPDAC followed by radiotherapy, and only one, (0.8%) was treated with ABVD followed by radiotherapy (Adam et al., 2021). To estimate overall survival (OS), the duration of follow-up of the patients was calculated by using the date of diagnosis and the date of death, or the last date of follow-up. Event-free survival (EFS) was estimated by using the date of diagnosis and date of relapse, date of death, or last date of follow-up. Kaplan Mayer's analysis with log-rank test was used to estimate the OS and EFS. A result with *P*-value <0.05 was considered statistically significant. Univariate Cox-regression was used to estimate the hazard ratio (HR), Wald test, and 95% confidence interval (95% CI), assumptions for proportional hazard were not violated. Accordingly, covariates with *P* value ≤0.1 in the univariate Cox-regression were used for multivariate Cox-regression analysis to evaluate the independent impact of a given prognostic factor on survival. 4-year OS was conducted for 71 cases of CHL and NLPHL. For the 9 -year survival analysis 68 CHL cases were included and 3 NLPHL cases were excluded.

2.16. Cut-offs

Since there is no consensus for validated cutoff points to predict the prognostic impact of immune cells in the tumor microenvironment (TME) of CHL on the disease outcome (Table 2), different percentiles (10%, 20%, 25%, 30%, 40%, 50%, 60% 70%, 75%, 80%, 90%) were tested as cut-points for FoxP3+ cells% and PD1+ cells% in the TME of CHL. These cut-points were

examined by Kaplan-Meier method to identify any significant prognostic impact of these biomarkers on the treatment outcome.

Table 2. Cutoff points for FoxP3 and PD1 in the TME of CHL used in previous studies

Biomarker/n	Method of quantification	Cutoff point	P value of OS	P value of EFS	Reference
FoxP3					
n= 87	Histo image analysis	500/mm^2	0.6	0.6	(Schreck et al., 2009)
n= 90	Ariol imaging system	<125/HPF low 125-500/HPF intermediate >500/HPF high	0.006	0.002	(Greaves et al., 2013)
n= 63		>1 (>10% of positive cells)	0.8	0.03	(Chetaille et al., 2009)
PD1					
n= 122	Ariol imaging system		0.012	-	(Greaves et al., 2013)
n= 63	Manual scoring	>1 (>10% of positive cells)	0.4	0.4	(Chetaille et al., 2009)

3. Results

3.1.Demographic and clinical characteristics of HL cases

3.1.1. Demographic characteristics of HL cases

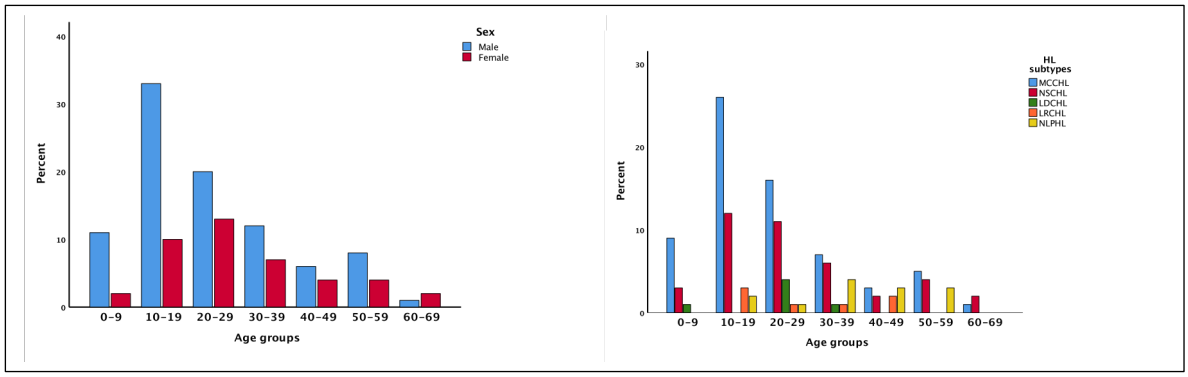
Out of a total of 133 HL patients, 68.4% (91) were males and 31.6% (42) were females, with a male to female ratio of 2.2:1 (*P* value < 0.001) (Table 3). The patients' age ranged between 4 and 66 with median age of 22 years. The finding showed that 9.8% (13), 32.3% (43), 24.6% (32), and 30.7% (40) of HL patients were children in the age groups (0-9 years), Adolescents (10-19 years), young adults (20-29 years) and adult (30-59 years), respectively. The most affected age groups in this study were adolescent (10-19 years) and young adults (20-29

years). The elderly (≥ 60 years) represented only 2.3% (3) of the cases. The risk of HL declined with increased age as shown in Figure 3a and Table 4. There was no significant difference in age distribution between male and female patients (*P*= 0.83). The majority of HL patients were from Oromia Regional State 35.2% (43), followed by Addis Ababa 27.9% (34) and Amhara Regional State 22.1% (27) as depicted in Table 5.

Table 3. Demographic and clinical characteristics of HL cases in this study diagnosed between 2014 and 2019 at TASH

Characteristic	**n**	**%**	**Characteristics**	**n**	**%**
Sex			**Ann Arbor stage**		
Male	91	68.4	Stage I	31	26.5
Female	42	31.6	Stage II	46	39.3
Total	133		Stage III	25	21.4
Age			Stage IV	15	12.8
0-9	13	9.8	**Total**	**117***	
10-19	43	32.3	**B-symptoms**		
20-29	33	24.8	Yes	73	54.9
30-39	19	14.3	No	60	45.1
			Total	133	
40-49	10	7.5	**HIV status**		
50-59	12	9	Positive	12	13
≥60	3	2.3	Negative	80	87
Total	133		Total	92	

*16 cases with missing stage.



(a) (b)

Figure 3. Hodgkin lymphoma distribution among the study participants, TASH, Addis Ababa, diagnosed between the years 2014 and 2019. (a) Age and sex distribution of HL patients included in the study (b) Hodgkin lymphoma subtypes among different age groups.

Table 4. The age and sex distribution of Hodgkin's lymphoma during the study period (2014-2019), at TASH

Characteristic	Sex				Total	
	Male		Female			
Age groups	**n**	**%**	**n**	**%**	**n**	**%**
0-9	10	7.5	3	2.2	13	9.1
10-19	35	26.3	10	7.5	45	34.1
20-29	20	15	12	9.2	32	24.2
30-39	11	8.3	7	5.2	18	13.6
40-49	6	4.5	4	3.0	10	7.6
50-59	8	6.1	4	3.0	12	9.1
60-69	1	0.7	2	1.5	3	2.3
Total	91	68.4	42	31.6	133	100

Table 5. Distribution of HL patients in Ethiopia during the study period (2014-2019), at TASH

Permanent Residence	**n**	**%**	**Population***
Addis Ababa	34	27.9	3,434,000
Amhara	27	22.1	21,134,988
Dire dawa	1	.8	466,000
Harrari	1	.8	246,000
Oromia	43	35.2	35,467,000

SNNPR	12	9.8	19,170,007
Somali	1	.8	5,748,998
Tigray	3	2.5	5,247,005
Total	122	100.0	
Missing	11		

*(CSA, 2007)

3.1.2. Clinical and Histopathological Pattern of HL in Ethiopia

The patients were presented with cervical lymphnodes enlargements at diagnosis and 15% (20), 7.5% (10), and 6% (8) of the patients were with axillary, supraclavicular, and inguinal lymphnodes involvement, respectively. Most of the patients, 54.9% (73), presented with one or more B-symptoms at diagnosis. According to the Ann Arbor staging system (Carbone et al., 1971); 26.5% (31), 39.3% (46), 21.4% (25), and 12.8% (15) of the patients respectively presented with stage I, II, III, and IV. 65.1% (84) of the patients presented with anemia (hemoglobin level of <13.7g/dl for males and <11.2g/dl for females were used as a *cut-off* for anemia), 61.8% (81) with lymphocytopenia (*cut-off* <1.18x10^3/µl) and 54.2% (71) with monocytopenia (*cut-off* <0.24x0^3/µl). MCCHL was the dominant histopathological subtype, 50.4% (67) followed by NSCHL 30.1% (40) as displayed in Table 6. The MCCHL subtype was more common in males 73.1% (49) than in females 26.9% (17) with a ratio of 2.6:1. The distribution of NSCHL subtype among sexes was almost similar, with a ratio of 1.2:1as depicted in Table 7. The median age of HL cases was different between the CHL subtype and the NLPHL, 21 and 38 years, respectively (*P* value of 0.004 and 95% CI 3.68-18.68). MCCHL and NSCHL were more common among patients up to 29 years of age, while NLPHL was more common among patients above 30 years of age (Figure 3b).

Table 6. Histopathological and hematological characteristics of HL cases included in the study, diagnosed between the years 2014 and 2019 at TASH

Characteristics	**N**	**%**	**Characteristics**	**n**	**%**
HL subtypes			Lymphocytes count at diagnosis		
MCCHL	67	50.4	>0.6x 10^3/µl	79	59.4
NSCHL	40	30.1	≤0.6x 10^3/µl	52	39.1
LDCHL	6	4.5	Haemoglobin at diagnosis g/dl		
LRCHL	7	5.3	>10.5	70	54
NLPHL	13	9.8	≤10.5	59	46
White blood cell (WBC) count at diagnosis			Monocyte count at diagnosis		
WBC≤ 15x10^3/µl	114	86.4	≥ 0.24	60	45.8
WBC>15x10^3/µl	18	13.6	< 0.24	71	54.2

Table 7. Proportion of Hodgkin's lymphoma subtypes among sex and age groups of HL cases diagnosed between the years 2014 and 2019 at TASH

Sex	**HL sub-types**									
	MCCHL		**NSCHL**		**LRCHL**		**LDCHL**		**NLPHL**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Male	49	73.1	23	57.5	6	85.7	4	66.7	9	69.2
Female	18	26.9	17	42.5	1	14.3	2	33.3	4	30.8
M:F ratio	**2.6:1**		**1.2:1**		**6:0**		**2:1**		**2:1**	
Age group										
0-9	9	13.4	3	7.5	0	0.0	1	16.7	0	0
10-19	26	38.8	12	30	3	42.9	0	0	2	15.4
20-29	16	23.9	11	27.5	1	14.3	4	66.7	1	7.3
30-39	7	10.4	6	15	1	14.3	1	16.7	4	30.8
40-49	3	4.5	2	5	2	28.6	0	0	3	23.1
50-59	5	7.5	4	10	0	0.0	0	0	3	23.1
60-69	1	1.5	2	5	0	0.0	0	0	0	0

3.1.3. HL therapeutic modalities in Ethiopia

The majority of HL patients 93.4% (124) were treated with the standard chemotherapy regimen ABVD only, while 6.8% (9) of the patients were treated with ABVD followed by cyclophosphamide, vincristine sulfate, prednisone, and dacarbazine (COPDAC). 3.8% (5) of the patients received ABVD and COPDAC followed by radiotherapy, and only 0.8% (1) was treated with ABVD followed by radiotherapy, Table 8. The number of ABVD treatment cycles for HL patients in this study ranged between 4-8 cycles. Three patients (2.3%) received < 4 cycles of ABVD, these patients stopped treatment and follow-up earlier than planned. The number of COPDAC cycles ranged between 2-6 cycles. 48.3% (14) of HL patients within the age group≤ 14 years were treated with the ABVD regimen only, while 51.7% (15) of this age group were treated with combined chemotherapy/radiotherapy. All HL cases within the age group >14 years were treated with ABVD. A total of 94.7% (36) and 5.3% (2) of HL cases with early-stage disease received ABVD only and combined chemotherapy/radiotherapy, respectively. 85.1% (74) and 14.9% (13) of HL cases with advanced stage disease received ABVD only and combined chemotherapy/radiotherapy, respectively.

Table 8. Treatment modalities and treatment cycles of HL cases included in the study, diagnosed between the years 2014 and 2019 at TASH

Therapeutic modalities	n	%	Characteristics	n	%
ABVD*	124	93.4	ABVD treatment cycles		
ABVD & COPDAC‡	9	6.8	< 4 cycles	3	2.3
ABVD, COPDAC & Radiotherapy	5	3.8	4 cycles	42	31.5
ABVD & Radiotherapy	1	0.8	6 cycles	55	41.4
Missing	9	6.8	8 cycles	17	12.8

Total	133	100	Total	117	88.0
COPDAC treatment cycles			Missing	16	12.0
2 cycles	11	8.3	Total	133	100.0
4 cycles	2	1.5			
6 cycles	1	.8			
None	119	89.5			
Total	133	100.0			

ABVD*: Adrimycin (doxorubicin), bleomycin, vinblastine, and dacarbazine), COPDAC‡:cyclophosphamide, vincristine sulfate, prednisone, and dacarbazine

3.2.Status of HL cases in relation to viral infection

3.2.1. HIV status of HL cases

HIV test results were available for 92 patients, and 13% (12) were positive. 63.7% (7) of the HIV-positive presented with stage I-II at diagnosis. However, 75% (9) of HIV-positive cases were categorized into the group of advanced stage, when re-staged using B-symptoms. The distribution of HIV-associated HL was similar between males and females. 83.3% (10) of HIV-associated HL cases were within the age group of >14 years while 60% (6) of HIV-associated HL cases were of MCCHL subtypes followed by NSCHL subtype 30% (3) as shown in Table 9. Monocytopenia was strongly associated with HIV as 75% (9) of HIV-associated HL presented with low monocyte counts at the time of diagnosis.

Table 9. Demographic and Clinical characteristics of HL patients according to HIV status, diagnosed between the years 2014 and 2019 at TASH

Clinical characteristics		**HIV Positive**	**HIV Negative**	**95%CI**	***P value***
		n (%)	**n (%)**		
HL Subtypes					0.219
	MCCHL	6(12)	44 (88)		
	NSCHL	3(10)	27 (90)		
	LRCHL	2 (50)	2 (50)		
	LDCHL	0	2 (100)		
	NLPHL	1 (20)	4 (80)		
Stage of the disease					0.485
	Stage I	4 (15.4)	22(84.6)		
	Stage II	3(12)	22 (88)		
	Stage III	1 (5.3)	18 (94.7)		
	Stage IV	3 (23.1)	10 (76.9)		
Early stage		3 (12.5)	21 (87.5)		
Advance stage		9 (13.4)	58 (86.6)		
Sex distribution				-0.05-0.28	0.172
	Male	6 (9.5)	57 (90.5)		
	Female	6 (22.2)	21 (77.8)		
Age groups				-0.04-0.22	0.331
	≤14	2 (7.1)	26 (92.9)		
	>14	10 (15.9)	53 (84.1)		
WBC count x 10^3/µl				(0.07-0.67)	**0.005**
	≥3.98	7 (8.8)	73 (91.3)		
	<3.98	5 (5.5)	6 (54.5)		
Lymphocyte count x				-0.007-0.26	0.069
10^3/µl	≥1.18	3 (6.7)	42 (93.3)		
	<1.18	9 (19.6)	37(80.4)		
Monocyte count x				0.04-0.34	**0.013**
10^3/µl	≥0.24	3 (5.6)	51 (94.4)		
	<0.24	9 (24.3)	28 (75.7)		
Hemoglobin g/dl				-0.22-0.10	0.51
	≥11.2	5 (17.2)	24 (82.8)		
	<11.2	7 (11.5)	54(88.5)		

3.2.2. Detection of LMP1 and EBER in the HRS cells of HL

In this study, EBV was detected only in CHL cases. A total of 77/126 (61.1%) of HL cases expressed LMP1 and EBER. EBV expression was higher in males (64/91, 70.3%) than in females (13/31, 41.9%), *P* = 0.012. As shown in Figure 4a and Table S5, the expression of LMP1 and EBER on HL tumor cells was higher in the pediatric population (≤14 years) and in younger adults (15–34 years) (*P* = 0.02). Among the two predominant CHL subtypes, LMP1 and EBER were detected in 48/65 (73.8%) and 23/40 (57.5%) cases of the MCCHL and NSCHL subtypes, respectively (Figure 4b, Table 10). The MCCHL subtype was highly associated with EBV, with a *P* value of <0.001. As shown in Table 10, HL cases presented in all disease stages, and the disease stage was not related to EBV. Furthermore, logistic regression analysis was conducted to investigate the independent relationship of HL subtypes, age, and sex with EBV. In this analysis, we found that male sex and MCCHL subtype were independently associated with EBV (Table 11).

Table 10. EBV association with clinicopathological characteristics of HL in Ethiopia, among HL cases diagnosed at TASH between the years 2014 and 2019

Characteristics	**LMP1 and EBER in tumor cells, n (%)**	***P****
EBV infection		0.013
Positive cases	77 (61.1)	
Negative cases	49 (38.9)	
Age groups (years)		0.020
0-14 (N = 42)	34 (44.2)	
15-24 (N = 41)	21 (27.3)	
25-34 (N = 17)	10 (13)	
35-44 (N = 7)	4 (5.2)	
≥45 (N = 18)	8 (10.4)	
Sex		0.012

Male (N = 91)	64 (83.1)		
Female (N = 31)	13 (16.9)		
Stage of the disease			0.261
Stage I (N = 32)	24 (75)		
Stage II (N = 48)	29 (60.4)		
Stage III (N = 25)	14 (56)		
Stage IV (N = 15)	8 (53.3)		
HL subtypes			<0.001
MCCHL (65)	48 (62.3)		
NSCHL (40)	23 (29.9)		
Others (11)	6 (7.8)		
NLPHL (10)	0		

*Chi-Squar (x^2) or Fisher exact test as appropriate

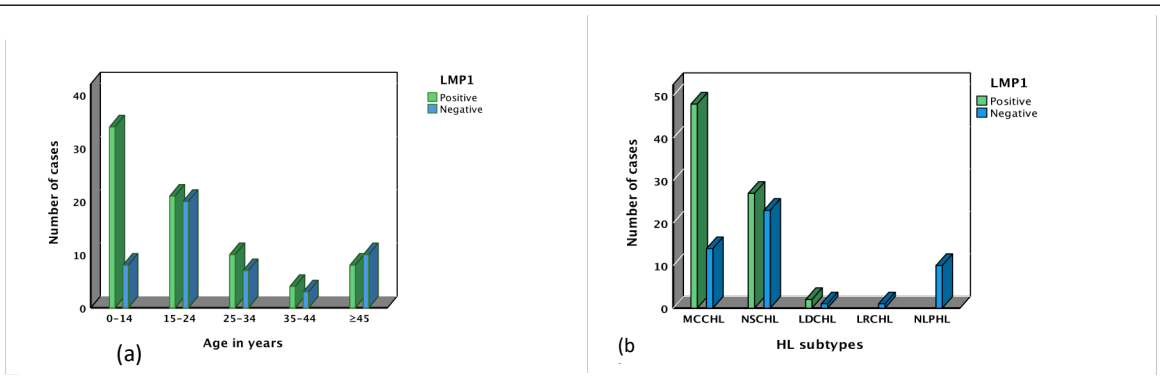


Figure 4. Association of age and histopathological subtypes of HL cases with EBV infection, TASH, cases diagnosed between 2014 and 2019. (a) association of age groups with EBV markers expression on HL ($P = 0.02$). (b) Differences in the expression of EBV markers between HL subtypes ($P< 0.001$)

Table 11. EBV association with the age, sex, and HL subtypes of CHL cases, diagnosed between 2014 and 2019 at TASH

EBV Marker/ Characteristics	**Wald x^2(1)**	**COR** (95% CI) *P**	**Wald x^2(1)**	**Adj OR*** (95% CI), *P****
n = 126				
EBV+				
Age	4.2	1.02 (1.001-1.06), 0.03	3.03	1.03 (0.9-1.05), 0.08
Sex	6.3	3 (1.3-7), 0.012	4.9	2.9 (1.1-7), 0.026

CHL subtypes[§]	5.05	2 (1.09-3.6), 0.025	6.6	2.3 (1.2-4.2), 0.01

*Logistic regression **COR: crude odds ratio, ***Adj OR: adjusted odds ratio, §CHL subtypes: MCCHL vs other subtypes

3.3. Immune cells in the tumor microenvironment of HL cases

3.3.1. Image analysis quantification and phenotype characterization of cell biomarkers in the TME of HL by IHC

Detailed descriptions of the count, percentage, mean and median of cell biomarkers in the TME of total HL cases (CHL and NLPHL) and the TME of CHL cases are presented in Tables 12A, 12B, and 12C. In the TME of the studied HL cases, the mean percentages of cells expressing CD3 and CD20 were 79.6% and 29% of the total cells/mm^2, respectively, P<0.001 (Figure 5a and Table 12D). The proportion of CD4+ cells, which was 55.9% of the total cells, was higher than the proportion of CD8+ cells (27.9%) (P <0.001). The CD4:CD8 ratio was 2:1 (Figure 5b and Table 12D). Furthermore, the biomarkers FoxP3, T-bet, and C-maf, which are associated with Treg, Th1, and Th2, were positive in 8.8%, 18.7%, and 24.4% of the total cells, respectively (Figure 5c). The proportion of cells expressing C-maf was higher than that of cells expressing T-bet, P = 0.004 (Figure 5d and Table 12D), and 5% of the total cells in the TME expressed the programmed death 1 (PD1) biomarker. PDL1 was expressed in 95% of HL cases.

Table 12. The Description of the quantified immune cells biomarker in the tumor microenvironment of HL by HALO image analysis software, the HL cases dignosed at TASH between the years 2014 and 2019

A. Description of the biomarker in the TME of all cases (NLPHL & CHL cases)

Biomarker (n)	**Total cells count/mm^2**		**Positive cells count/mm^2**	
	Mean (SE)	**Std deviation (range)**	**Mean (SE)**	**Std. deviation (Range)**
CD20+ (126)	7961 (390)	4386 (141-17135)	2319 (214)	2406 (1-11984)
CD3+ (120)	12019 (264)	2895 (4613-21078)	9163 (338)	3707 (747-16310)
CD4+ (120)	12635 (308)	3381 (4460-22671)	6654 (431)	4725 (17-19809)
CD8+ (120)	11742 (286)	3134 (4121-22043)	3738 (263)	2882 (109-12870)
FoxP3+ (117)	12458 (270)	2962 (3723-20801)	1181 (90)	988 (27-4792)
T-bet+ (119)	12501 (277)	3029 (4868-19966)	2669 (200)	2190 (14-9295)
C-maf + (118)	12162 (273)	2971 (3734-19460)	3227 (171)	1863 (228-10891)
PD1+ (113)	12731 (296)	3156 (2906-19976)	2051 (269)	2860 (0-15872)

B. Description of the expressed biomarkers in the TME of CHL (positive cells count/mm^2)

Biomarker (n)	**Positive cells count /mm^2**		**Percentile of cells count/mm^2**		
	Median	**Range**	**25%**	**50%**	**75%**
CD20+ (N = 112)	1447	1-9721	311	1447	3730
CD3+ (N = 107)	9031	747-15486	6714	9031	11606
CD4+ (N = 107)	5343	17-18683	2230	5343	10357
CD8+ (N = 107)	3119	109-12870	1366	3119	5030
FoxP3+ (N = 107)	941	66-4792	479	941	1938
T-bet+ (N=106)	2289	14-9295	827	2289	4175
C-maf +(N=105)	2778	228-10891	1818	2778	4496
PD1+ (N=102)	621	0-9337	208	621	2254

C. Description of the expressed biomarkers in the TME of CHL (percent of positive cells/mm^2)

Biomarker (n)	**Percent of positive cells /mm^2**		**Percentile**		
	Median	**Range**	**25%**	**50%**	**75%**
CD30+ (116)	72.5	5-95	57	72	85
CD20+ (112)	29	0-99	5.6	28	72.9
CD3+ (107)	79.6	15-99	63.7	79.6	90
CD4+ (107)	52	0-99	20.2	52.6	75
CD8+ (107)	27.9	1-95	11.9	27.9	46.5
FoxP3+ (107)	8.8	1-35	3.8	8.8	15

T-bet+ (106)	18.7	0-77	8	18.7	34.7
C-maf + (105)	24.4	1-78	16.6	24.4	37
PD1+ (102)	5	0-64	1.6	5	16.6

D. The differences between the proportion/mm^2 of biomarkers expression in the TME of HL cases (all cases)

Biomarker	**Cell count/mm^2**	**Percent/mm^2**
	P*	P
CD3 vs CD20	<0.001	0.036
CD4 vs CD8	<0.001	<0.001
C-maf vs T-bet	0.029	0.004

*Wilcoxon Signed Rank test

A positive linear correlation was detected between the proportion of PD1+ cells and the proportion of CD4+ cells (P <0.001). The quantity of PD1+ cells was also positively correlated with that of C-maf+ cells (P = 0.039). In contrast, the PD1 expression level showed a negative correlation with the FoxP3 expression level (P=0.002), (Table 13A). The number of FoxP3+ cells showed a positive correlation with the number of cells expressing CD8 and T-bet biomarkers, with P-values of 0.014 and 0.01, respectively (Table 13B). A positive correlation was observed between the expression of CD8 and T-bet biomarkers (P< 0.001), (Table 13C)

Table 13. Correlation of biomarkers expressed in the cells of the TME of HL cases diagnosed at TASH between the years 2014 and 2019

A. Correlations between the inhibitory checkpoint marker expression and the T-cell biomarkers in the TME of HL (all cases)

Biomarkers	**Spearman's rho**	***P* value**	**95% CI**
PD1 with CD4	0.497	<0.001	0.34-0.62
PD1 with CD8	-0.032	0.735	-0.22-0.16
PD1 with FoxP3	-0.289	0.002	-0.45—0.1
PD1 with T-bet	-0.038	0.876	-0.23-.015
PD1 with C-maf	0.195	0.039	0.005-0.37

B. FoxP3 correlations with CD8, T-bet, and C-maf biomarkers expressed by the cells of the TME of HL

Biomarkers	**Spearman's rho**	**P value**	**95% CI**
FoxP3 with CD8	0.225	0.014	0.04-0.39
FoxP3 with T-bet	0.235	0.01	0.052-0.403
FoxP3 with C-maf	0.177	0.056	-0.01-0.35

C. Correlations of CD8-expression with the expression of T-bet on the cells of the TME of HL

Biomarker	**Spearman's rho**	**P value**	**95% CI**
C8 with T-bet	0.396	<0.001	0.22-0.54

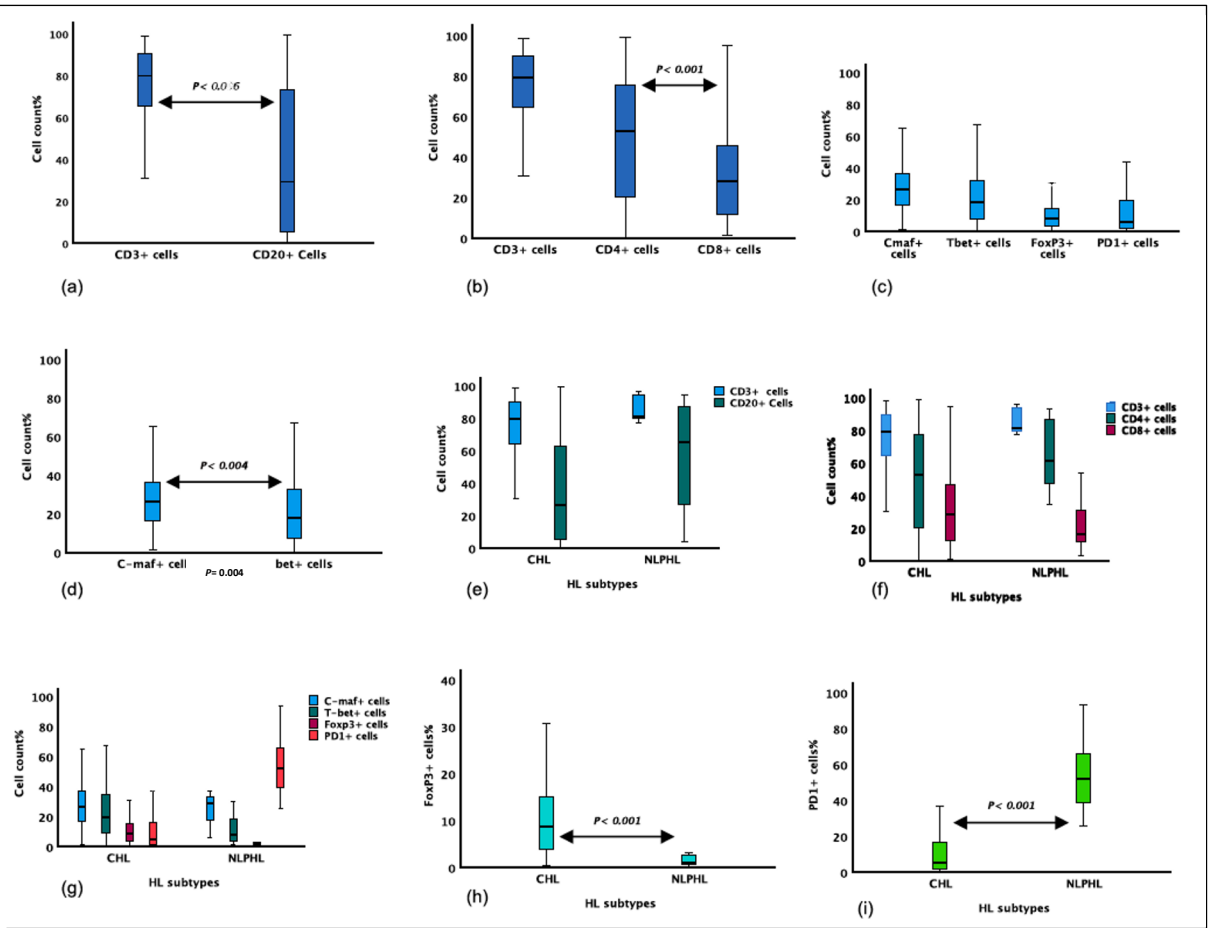


Figure 5. Mean differences of immune markers expression on the cells of the tumor microenvironment of HL cases diagnosed between 2014 and 2019 at TASH. Panel 'a' through 'd' represent data of all HL cases (CHL & NLPHL); (a) mean differences between CD3+ and CD20+ cells, (b) proportion of CD3, CD4, and CD8 and the mean difference between CD4+ and CD8+ cells, (c) proportion of T-cell subsets markers; C-maf, T-bet, FoxP3 and PD1, (d) mean difference between C-maf+ and T-bet+ cells. Panel e through i show differences of immune markers expression between cHL & NLPHL; (e) proportion of CD3+ and CD20+ cells in CHL and NLPHL subtypes, (f) proportion of CD3+, CD4+ and CD8+ cells in CHL and NLPHL subtypes, (g) proportion of C-maf+, T-bet+, FoxP3+ and PD1+ cells in CHL and NLPHL, (h) Difference of FoxP3+ cells between CHL and NLPHL, (i) Difference of PD1+ cells between CHL and NLPHL. P values are depicted where statistically significant

3.3.2. The association of immune cells in the tumor microenvironment of HL with clinicopathological features

The proportion of CD20+ and CD3+ cells was higher in the NLPHL type than in the CHL type (Figure 5e, Tables 14A and 14B). The proportion of CD4+ cells/mm^2 was also higher in NLPHL cases than in CHL cases (*P* = 0.19) (Figure 5f, Tables 14A and 14B). The proportion of cells expressing CD8 was numerically higher in CHL cases than in NLPHL cases, but the difference was not significant. The CD4:CD8 ratio was higher in NLPHL, with a *P* value of 0.05. Moreover, FoxP3 expression was higher in CHL than in NLPHL (*P* = <0.001) (Figure 5g & h). The cells in the TME of both HL types showed no differences in the expression of the Th1 and Th2 biomarkers T-bet and C-maf (Figure 5g). The proportion of cells expressing the immune checkpoint marker PD1 was markedly higher in NLPHL (*P* <0.001) (Figure 5i, Tables 14A and 14B)

Table 14. The association of biomarkers expression with the two subtypes of HL (CHL & NLPHL) cases diagnosed at TASH between the years 2014 and 2019

A. The mean differences of immune cell biomarkers count/mm^2 between HL types

Immune marker (N)	**Mean (Std. error)**	***t* (95% CI), *P****	***P*** **
CD20		0.9 (-892-2250), 0.4	0.5
NLPHL (10)	2944 (1129)		
CHL (116)	2265 (212)		
CD3		2.4 (517-5507), 0.018	0.02
NLPHL (9)	11950 (1127)		
CHL (111)	8937 (346)		
CD4		2 (148-6547), 0.04	0.045
NLPHL (9)	9761 (1644)		
CHL (111)	6413 (440)		
CD8		0.6 (-1372-2595), 0.5	0.7

NLPHL (9)	3142 (700)		
CHL (111)	3754 (278)		
FoxP3		6.2 (618-1235), <0.001	<0.001
NLPHL (9)	324 (114)		
CHL (111)	1251 (94)		
T-bet		1.2 (-619-2383), 0.25	0.28
NLPHL (9)	1853 (513)		
CHL (110)	2735 (212)		
C-maf		0.5 (-952-1615), 0.6	0.5
NLPHL (9)	3534 (672)		
CHL (109)	3202 (177)		
PD1		4 (2848-11042), 0.006	<0.001
NLPHL (7)	8567 (1673)		
CHL (106)	1621 (208)		

*The mean differences were analyzed using an independent t-test, ** Non-parametric Mann-Whitney test

B. The mean differences of immune cell biomarkers percentage/mm^2 between HL types

Immune marker (n)	**Mean (Std. error)**	***t* (95% CI), *P****	***P*** **
CD20		1.8 (-2-42), 0.07	0.07
NLPHL (10)	57 (10)		
CHL (116)	37 (3)		
CD3		1.3 (-4-23), 0.19	0.18
NLPHL (9)	82 (4)		
CHL (111)	73 (2)		
CD4		1.3 (-6-35), 0.17	0.19
NLPHL (9)	64 (7)		
CHL (111)	49 (2)		
CD8		1.2 (-6-24), 0.2	0.2
NLPHL (9)	22 (5)		
CHL (111)	32 (2)		
FoxP3		6.8 (5-10), <0.001	<0.001

NLPHL (9)	2 (0.9)		
CHL (111)	10 (0.7)		
T-bet		1.6 (-1-21), 0.1	0.09
NLPHL (9)	12 (3)		
CHL (110)	22 (1)		
C-maf		0.8 (-5-13), 0.4	0.5
NLPHL (9)	23 (4)		
CHL (109)	27 (1)		
PD1		6.5 (29-54), <0.001	<0.001
NLPHL (7)	54 (8)		
CHL (106)	12 (1)		

*The mean differences analyzed using independent t-test, ** Non-parametric Mann-Whitney test

In a linear regression model, NLPHL was independently associated with a high proportion of PD1+ cell count compared to the CHL type. In contrast, the proportion of FoxP3+ cells was higher in CHL than in NLPHL. Age and sex were not associated with CD3, CD4, FoxP3 or PD1 expression. However, the quantity of C-maf+ cells was higher among elderly cases, *P* = 0.03. (Table 15).

Table 15. Association of the clinical characteristics of HL with the expression of the biomarkers in the TME of HL cases (both NLPHL and CHL cases), diagnosed at TASH between the years 2014 and 2019

Biomarker/clinical characteristic	**Adj OR (95% CI), *P* ***	
	Biomarker count/mm^2	**Biomarker percent/mm^2**
CD3 proportion		
HL classes**	2.4 (503-5649), 0.02	1.4 (-4-24), 0.16
Age	0.13 (-44-50), 0.9	-0.5 (-0.3-0.2),0.6
Sex	0.6 (-1090-2135), 0.5	-0.4 (-11-7), 0.7
CD4 proportion		
HL classes	2 (46-6601), 0.047	1.4 (-6-36), 0.16

Age	0.6 (-43-77), 0.6	0.2 (-0.35-0.42), 0.8
Sex	1.2 (-856-3302), 0.2	1.01 (-6-20), 0.3
CD8 proportion		
HL classes	-0.5 (-2488-1458), 0.6	-1.1 (-24-6), 0.3
Age	-0.3 (-42-30), 0.8	-0.3 (-0.3-0.23), 0.7
Sex	1.7 (-2264-203), 0.1	2 (0.08-19), 0.05
FoxP3 proportion		
HL classes	-2.9 (-1642-(-304)), 0.005	-3.2 (-13-(-3)), 0.002
Age	1.09 (-5-19), 0.2	1.3 (-0.03-0.16), 0.2
Sex	1.5 (-93-743), 0.1	1.5 (-0.78-5), 0.13
T-bet proportion		
HL classes	-1.3 (-2576-510), 0.18	-1.9 (-22-0.75), 0.07
Age	1.3 (-10-46),0.2	1.4 (-0.06-0.37), 0.16
Sex	0.8 (-581-1377), 0.4	1.16 (-3-11), 0.2
C-maf proportion		
HL classes	0.12 (-1221-1380), 0.9	-1.2 (-16-3), 0.2
Age	2.2 (2-50), 0.03	2.09 (0.01-0.38), 0.04
Sex	0.08 (-791-860), 0.9	0.37 (-5-7), 0.7
PD1 proportion		
HL cases	7.3 (4918-8588), <0.001	6.2 (27-54), <0.001
Age	1.5 (-7-55), 0.13	1.3 (-0.08-0.37), 0.2
Sex	0.12 (-1012-1145), 0.9	-0.7 (-10-4), 0.5

*Linear regression, **HL classes are the CHL and NLPHL

3.3.3. The proportion of immune cells in the microenvironment of EBV-related and EBV-unrelated CHL

There were no differences in the proportions of CD20+, CD3+, and CD4+ cells between the TME of EBV-related and EBV-unrelated CHL (Figure 6a & b). However, the proportion of CD8+ cells in the TME of CHL was significantly associated with EBV infection (*P* <0.001) (Figure 6c, Tables 16A and 16B). Accordingly, the ratio of CD4:CD8 was 3.4 (2-7) times higher in the EBV-

unrelated cases, *P* = 0.001, Figure 6d. The finding showed no difference in the proportion of C-maf+ cells between EBV-related and EBV-unrelated cases (Figure 6e & f). In contrast, the proportion of T-bet+ cells was significantly associated with EBV-related CHL (*P* <0.001, Figure 6e & g). EBV-related CHL was also associated with a higher proportion of cells expressing FoxP3 (*P* = 0.016, Figure 6e & h) and Figure 7. In contrast, the expression of PD1 was higher in EBV-unrelated CHL (*P* = <0.001). (Figure 6e & i, Tables 16A and 16B).

Table 16. The association of biomarkers expression with EBV-related and EBV-unrelated CHL cases, diagnosed at TASH between the years 2014 and 2019

A. Count of cells expressing biomarkers/mm^2

Immune marker (n)	**Mean (Std. error)**	***t* (95% CI), *P****	***P*** **
CD20			
LMP1 and EBER expression		1.3 (-297-1521), 0.2	0.07
EBV positive (73)	2493 (274)		
EBV negative (39)	1881 (359)		
CD3			
LMP1 and EBER expression		0.8 (-844-2072), 0.4	0.5
EBV positive (70)	9145 (414)		
EBV negative (37)	8531 (639)		
CD4			
LMP1 and EBER expression		0.5 (-1462-2323), 0.7	0.7
EBV positive (71)	6234 (538)		
EBV negative (36)	6665 (819)		
CD4:CD8			
LMP1 and EBER expression		3.4 (2-7), 0.001	0.002
EBV positive (70)	2.2 (0.3)		
EBV negative (36)	7.2 (1.9)		
CD8			
LMP1 and EBER expression		3.1 (623-2923), 0.003	<0.001
EBV positive (70)	4334 (350)		

EBV negative (37)	2560 (444)		
FoxP3			
LMP1 and EBER expression		2.5 (100-899), 0.015	0.018
EBV positive (71)	1391 (126)		
EBV negative (34)	891 (121)		
T-bet			
LMP1 and EBER expression		2.9 (451-2182), 0.003	<0.001
EBV positive (71)	3164 (265)		
EBV negative (35)	1847 (343)		
C-maf			
LMP1 and EBER expression		1.5 (-197-1339), 0.1	0.1
LMP1 positive (70)	3024 (204)		
LMP1 negative (35)	3595 (364)		
PD1			
LMP1 and EBER expression		3.08 (354-2375), 0.001	0.001
EBV positive (70)	1199 (210)		
EBV negative (32)	2564 (452)		

*The mean differences were analyzed using an independent t-test, ** Non-parametric Mann-Whitney test

B. Percent of cells expressing biomarkers out of the total cells/mm^2

Immune marker (n)	**Mean (Std. error)**	***t* (95% CI), *P****	***P*** **
CD20			
LMP1 and EBER expression		0.6 (-9-17), 0.6	0.4
EBV positive (73)	39 (3)		
EBV negative (39)	35 (5)		
CD3			
LMP1 and EBER expression		0.7 (-5-12), 0.4	0.8
EBV positive (70)	74 (2)		
EBV negative (37)	71 (4)		
CD4			
LMP1 and EBER expression		0.5 (-9-15), 0.6	0.5
LMP1 positive (71)	48 (3)		
LMP1 negative (36)	51 (5)		

CD8			
LMP1 and EBER expression		3.3 (6-23), 0.001	<0.001
EBV positive (70)	37 (2)		
EBV negative (37)	22 (3)		
FoxP3			
LMP1 and EBER expression		2.8 (1.09-6), 0.007	0.016
EBV positive (71)	10 (0.9)		
EBV negative (34)	7 (1)		
T-bet			
LMP1 and EBER expression		3.5 (4-17), <0.001	<0.001
EBV positive (71)	26 (2)		
EBV negative (35)	14 (2)		
C-maf			
LMP1 and EBER expression		1.9 (-0.08-13), 0.06	0.05
EBV positive (70)	25 (1)		
EBV negative (35)	31 (2)		
PD1			
LMP1 and EBER expression		2.6 (7-17), 0.01	<0.001
EBV positive (70)	9 (1)		
EBV negative (32)	19 (3)		

*The mean differences analyzed using independent t-test, ** Non-parametric Mann-Whitney test

Linear regression analysis was conducted for CHL, age, sex and EBV infection to detect the independent association of these variables with the proportion of cell biomarkers in the TME of CHL cases. The biomarkers with a *P* value ≤ 0.25 (in Table 16) were entered into the linear logistic regression model for multivariate analysis. EBV infection was independently associated with a high proportion of CD8+, FoxP3+ and T-bet+ cells. The FoxP3 association with EBV was still apparent (*P*= 0.03) after correcting for CD8 and T-bet in a linear logistic regression. In contrast, the proportion of PD1+ immune cells was higher among EBV-

unrelated cases (*P* = 0.02) (Table 17). In addition, the quantity of FoxP3+ cells was higher among males, *P* = 0.03 (Table 17).

Table 17. The association of clinical characteristics of HL with the expression of biomarkers in the TME of CHL, diagnosed between the years 2014 and 2019 at TASH

Biomarker/HL characteristics	**Count/mm2**	**Perecent/mm2**
	Adj OR (95% CI), P*	Adj OR (95%CI), P*
CD8		
EBV+	2.3 (183-2625), 0.025	2.7 (2-21), 0.012
Sex	-1.4 (-2310-397), 0.2	-1.5 (-18-2), 0.1
CHL subtype[§]	-1.3 (-1351-301), 0.2	-0.9 (-9-3), 0.4
FoxP3		
EBV+	3 (237-1094), 0.003	2.1 (0.16-7), 0.03
Sex	2.2 (47-958), 0.03	2.3 (0.5-8), 0.026
CHL subtypes[§]	-0.9 (-414-148), 0.4	-0.3 (-2.6-1.9), 0.7
Age	1.8 (-1-27), 0.08	1.6 (-0.02-0.2), 0.1
T-bet		
EBV+	3.5 (709-2578), <0.001	4 (7-21), <0.001
Age	2.3 (5-70), 0.021	2.5 (0.07-0.56), 0.014
PD1		
EBV+	-2.3 (-2030-(-162)), 0.02	-2.3 (-15-(-1)), 0.02
Age	1.8 (-2-60), 0.07	1.2 (-0.1-0.4), 0.3

*Linear regression, [§]CHL subtypes: MCCHL vs other subtypes

Expression of biomarkers on the TME cells in EBV-negative CHL was compared to the TME of NLPHL, showing a higher expression of FoxP3 and PD1 in the TME of EBV-negative CHL (*P* = 0.05) and NLPHL (*P* < 0.001), respectively as presented in Tables 18A and 18B.

Table 18. The differences in biomarkers expression on the TME immune cells of NLPHL and EBV-unrelated CHL cases, diagnosed between the years 2014 and 2019 at TASH

A. Count of cells expressing biomarkers/mm^2

Immune marker (n)	**Mean (Std. error)**	***t* (95% CI), *P****	***P***
CD20		1.2 (-795-2884), 0.3	0.2

NLPHL (10)	2944 (1129)		
EBV-negative CHL (39)	1881 (359)		
CD3		2.4 (571-6266), 0.02	0.018
NLPHL (9)	11950 (1127)		
EBV-negative CHL (37)	8531 (639)		
CD4		1.7 (-601-6794), 0.09	0.06
NLPHL (9)	9761 (1644)		
EBV-negative CHL (36)	6665 (819)		
CD8		0.6 (-1370-2534), 0.5	0.2
NLPHL (9)	3142 (700)		
EBV-negative CHL (37)	2560 (444)		
FoxP3		2.3 (72-1062), 0.02	0.08
NLPHL (9)	324 (114)		
EBV-negative CHL (34)	891 (121)		
T-bet		0.009 (-1463-1475), 0.9	0.6
NLPHL (9)	1853 (513)		
EBV-negative CHL (35)	1847 (343)		
C-maf		0.08 (-1669-1546), 0.9	0.9
NLPHL (9)	3534 (672)		
EBV-negative CHL (35)	3595 (364)		
PD1		4 (3513-8491), <0.001	<0.001
NLPHL (7)	8567 (1673)		
EBV-negative CHL (32)	2564 (452)		

B. Percent of cells expressing biomarkers out of the total cells/mm^2

Immune marker (n)	Mean (Std. error)	*t* (95% CI), *P**	*P***
CD20		1.7 (-3-47), 0.08	0.07
NLPHL (10)	57 (10)		
EBV-negative CHL (39)	35 (5)		
CD3		1.8 (-1-24), 0.07	0.3
NLPHL (9)	82 (4)		
EBV-negative CHL (37)	71 (4)		
CD4		1.4 (-5-31), 0.1	0.2
NLPHL (9)	64 (7)		
EBV-negative CHL (36)	51 (5)		
CD8		0.1 (-14-16), 0.7	0.5
NLPHL (9)	22 (5)		
EBV-negative CHL (37)	22 (3)		
FoxP3		3.5 (2-7), 0.001	0.05
NLPHL (9)	2 (0.9)		
EBV-negative CHL (34)	7 (1)		
T-bet		0.3 (-8-12), 0.7	0.8
NLPHL (9)	12 (3)		
EBV-negative CHL (35)	14 (2)		
C-maf		1.3 (-3-20), 0.1	0.1
NLPHL (9)	23 (4)		
EBV-negative CHL (35)	31 (2)		
PD1		4.3 (18-51), <0.001	<0.001
NLPHL (7)	54 (8)		
EBV-negative CHL (32)	19 (3)		

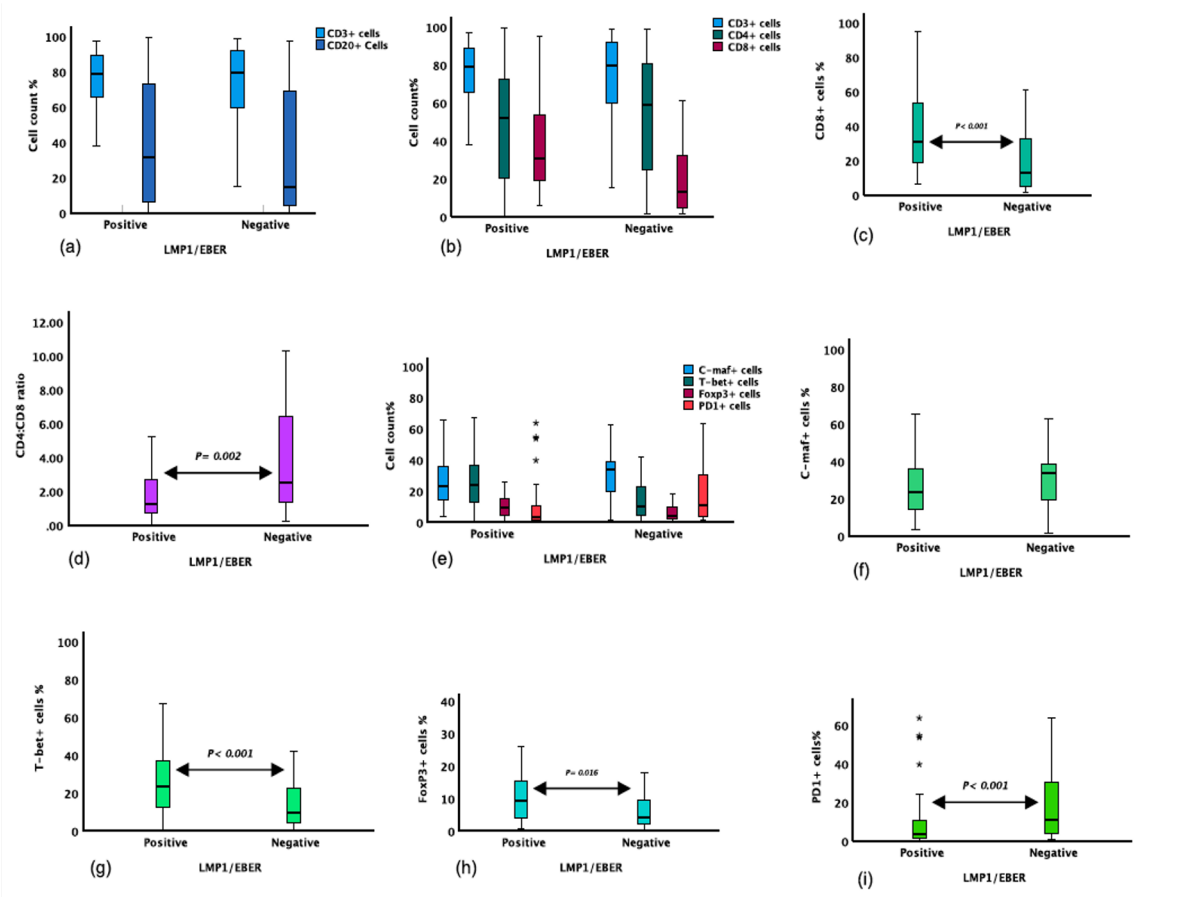


Figure 6. Mean differences of immune markers expression on the cells of the microenvironment (TME) of EBV-related and EBV-unrelated CHL cases, diagnosed between the years 2014 and 2019 at TASH. (a) Proportion of CD3+ and CD20+ cells, (b) Proportion of CD3+, CD4+ and CD8+ cell, (c) Difference in CD8+ cells proportion in the TME of the two groups, (d) Difference between EBV-related and EBV-unrelated CHL in CD4:CD8 ratio, (e) Proportion of C-maf+, T-bet+, FoxP3+ and PD1+ cells, (f) C-maf+ cells proportion in the TME of the two groups, (g) Difference in T-bet+ cells proportion between the two groups, (h) Difference in FoxP3+ cells proportion between the two groups, (i) Difference in PD1+ cells proportion in the TME between the two groups. P values are depicted where statistically significant. Data from panels f through i represent the same data from panel e but re-stratified by marker.

EBER: Epstein Barr Virus encoding-RNA, LMP1: Latent membrane protein

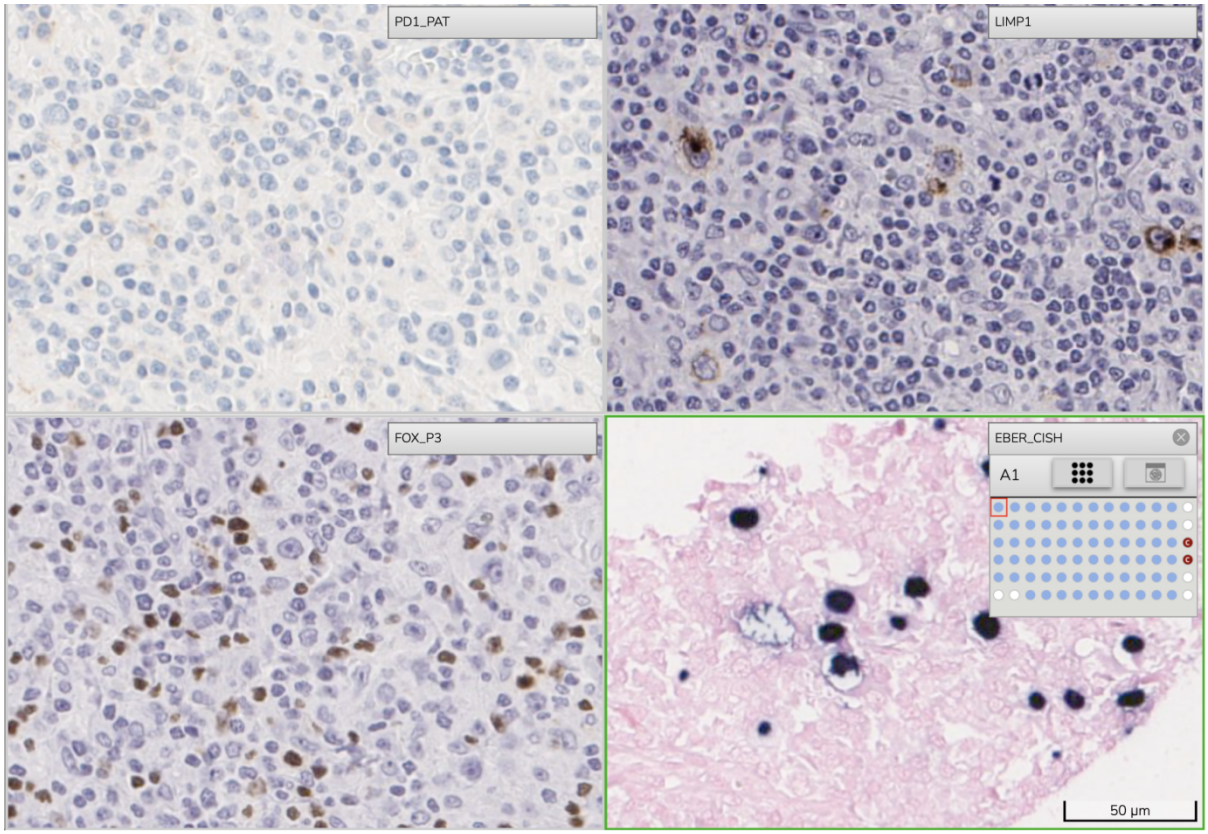


Figure 7. Slides for the same specimen of CHL case show that, LMP1 and EBER were expressed on the tumor cells, and the abundance of FoxP3+ and PD1+ cells in the tumor microenvironment of CHL was

3.4. Detection of Insulin-like growth factor II mRNA protein 3 (IMP3) in HL

3.4.1. Evaluation of the immune markers used for HL diagnosis compared to IMP3

In this study, the expression level was 96.8% for CD30 (122/126 were positive). All cases negative for CD30 were of the NLPHL subtype. The expression of CD15 and PAX5 were 95 (75.4%) and 121 (96%) respectively. 116 (92%) of the cases were negative for CD45 and 77 (61.1%) of the cases were positive for LMP1. Twenty-six (23%) of the cases were positive for CD20. All cases of NLPHL subtypes (10 cases) were strongly positive for CD20 and CD45 while the rest of the CD20 positive cases belonged to CHL subtypes (16 cases) with weak positivity for CD20 and negativity for CD45.

We then compared the expression of IMP3 with the immune markers currently used for the differential diagnosis of HL. Accordingly, CD30 was expressed on 122 (96.8%) of which 6 and 116 cases were of the NLPHL and CHL subtypes respectively, CD15 was expressed on 95 (75.4%), PAX-5 was expressed on 121 (96%) and IMP3 was expressed on 126 (100%) of the cases, as summarized in Table 19.

Table 19. Immunophenotypic characteristics of the HL tumor cells of the studied cases (n=126), diagnosed between 2014 and 2019 at TASH

Immunomarkers	**Negative n (%)**		**Positive n (%)**	
	CHL	NLPHL	CHL	NLPHL
IMP3	0	0	116 (92%)	10 (8)
CD30	0	4 (3.2%)	116 (92%)	6 (4.8)
CD15	17 (13.5%)	5 (4%)	99 (78.5%)	5 (4%)
CD20	99 (78.6%)	0	17 (13.5%)	10 (7.9)
CD45	116 (92%)	0	0	10 (8)
PAX5	5 (4%)	0	111 (88)	10 (8)
EBV+	39 (31%)	10 (7.9%)	77 (6 1.1)	0

The staining intensity of the immune markers CD30 and IMP3 on the tumor cells of HL was evaluated as weak, moderate, and strong (Figure 8). For CD30 staining intensity, 29 (23%), 38 (30.2%), and 55 (43.2%) of the cases were stained as weak, moderate, and strong, respectively. For IMP3 staining intensity, 21 (16.5%), 39 (30.7%), and 66 (52%) of the cases were stained as weak, moderate, and strong respectively, as indicated in Tables 20 and 21.

Table 20. IMP3 expressions on the HRS and LP cells in relation to clinical characteristics (n=126), diagnosed between 2014 and 2019 at TASH

Characteristics	Negative staining	Positive immunostaining intensity			*P* value
	n (%)	Weak n (%)	Moderate n (%)	Strong n (%)	
IMP3	0	21 (16.5)	39 (30.7)	66 (52)	
CD30	4 (3.2)	29 (23)	38 (30.2)	55 (43.6)	
IMP3 on HRS and LP Cells					
Sex					0.512
Male		18 (18.9)	28 (29.5)	49 (51.6)	
Female		3 (9.7)	11 (35.5)	17 (54.8)	
Age					
≤45		17 (15.6)	34 (31.2)	58 (53.2)	0.516
>45		4 (23.5)	5 (29.4)	8 (8.9)	
HL subtypes					0.007
MCCHL		10 (16.1)	13 (21)	39 (62.9)	
NSCHL		6 (12)	24 (48)	20 (40.3)	
LDCHL		0	1 (2.6)	2, (3)	
LRCHL		0	0	1 (1.5)	
NLPHL		5 (50)	1 (10)	4 (40)	
HL Classes					0.017
CHL		16 (13.8)	38 (32.8)	62 (53.4)	
NLPHL		5 (50)	1 (10)	4 (40)	
EBV					0.021
Positive		9 (11.7)	22 (28.6)	46 (59.7)	
Negative		12 (24.5)	17 (34.7)	20 (40.8)	
HIV status					0.066
Positive		0	0	4 (100)	
Negative		21 (17.2)	39 (32)	62 (50.8)	

3.4.2. Relation between IMP3 and CD30 expression and the clinicopathological features in HL

The expression level of IMP3 and CD30 was similar among the different sex and age categories. All NLPHL cases were positive for IMP3, but 5/10 (50%), showed only weak expression. The HRS cells of CHL expressed higher intensity of IMP3 and CD30 compared to the NLPHL. The differences in the biomarker expression according to subtype were significant with a *P* value of 0.017 for IMP3 and <0.001 for CD30 as depicted in Table 20 and Table 21, respectively. However, in the ordinal logistic model, no association was detected between any of the five HL subtypes and IMP3 stain intensity. A significant association was detected between the HL subtypes and CD30 staining intensity, with an odds ratio of 1.628 (95% CI, 1.026 to 2.584), Wald x^2 = 4.279, *P* = 0.039. No association was detected between CD30 and IMP3 staining intensity with HIV infection. High staining intensity of CD30 and IMP3 were both associated with EBV infection with an odds ratio of 4.246 (95% CI, 2.032 to 8.875), Wald $x^2(1)$ = 14.779, *P*< 0.001 for CD30; and an odds ratio of 2.305 (95% CI, 1.098 to 4.840), Wald $x^2(1)$ = 4.870, *P* = 0.027 for IMP3, (Table 22).

Table 21. CD30 expression on the HRS and LP cells in relation to clinical characteristics (n=126), diagnosed between 2014 and 2019 at TASH

Characteristics	Negative staining	Positive immunostaining intensity			*P* value
	n (%)	Weak n (%)	Moderate n (%)	Strong n (%)	
CD30	4 (3.2)	29 (23)	38 (30.2)	55 (43.6)	
CD30 on RHS and LP cells					
Sex					0.621
Male	2 (2.1)	23 (24.2)	29 (30.5)	41 (43.2)	
Female	2 (6.5)	6 (19.4)	9 (29)	14 (45.2)	
Age					0.276

≥45	2 (1.8)	25 (22.9)	33 (30.3)	49 (45)	
<45	2 (11.8)	4 (23.5)	5 (29.4)	6 (35.3)	
HL subtypes					<0.001
MCCHL	0	9 (14.5)	23 (37.1.5)	30 (48.4)	
NSCHL	0	13 (26)	14 (28)	23 (46)	
LDCHL	0	0	1 (33.3)	2 (66.7)	
LRCHL	0	1 (100)	0	0	
NLPHL	4 (40)	6 (60)	0	0	
HL Classes					<0.001
CHL	0	23 (19.8)	38 (32.8)	55 (47.4)	
NLPHL	4 (40)	6 (60)	0	o	
EBV					<0.001
Positive	0	11 (14.3)	24 (31.2)	42 (54.5)	
Negative	4(8.2)	18 (36.7)	14 (28.6)	13 (26.5)	
HIV status					0.467
Positive	0	0	2 (50)	2 (50)	
Negative	4 (3.3)	29 (23.8)	36 (29.5)	53 (43.4)	

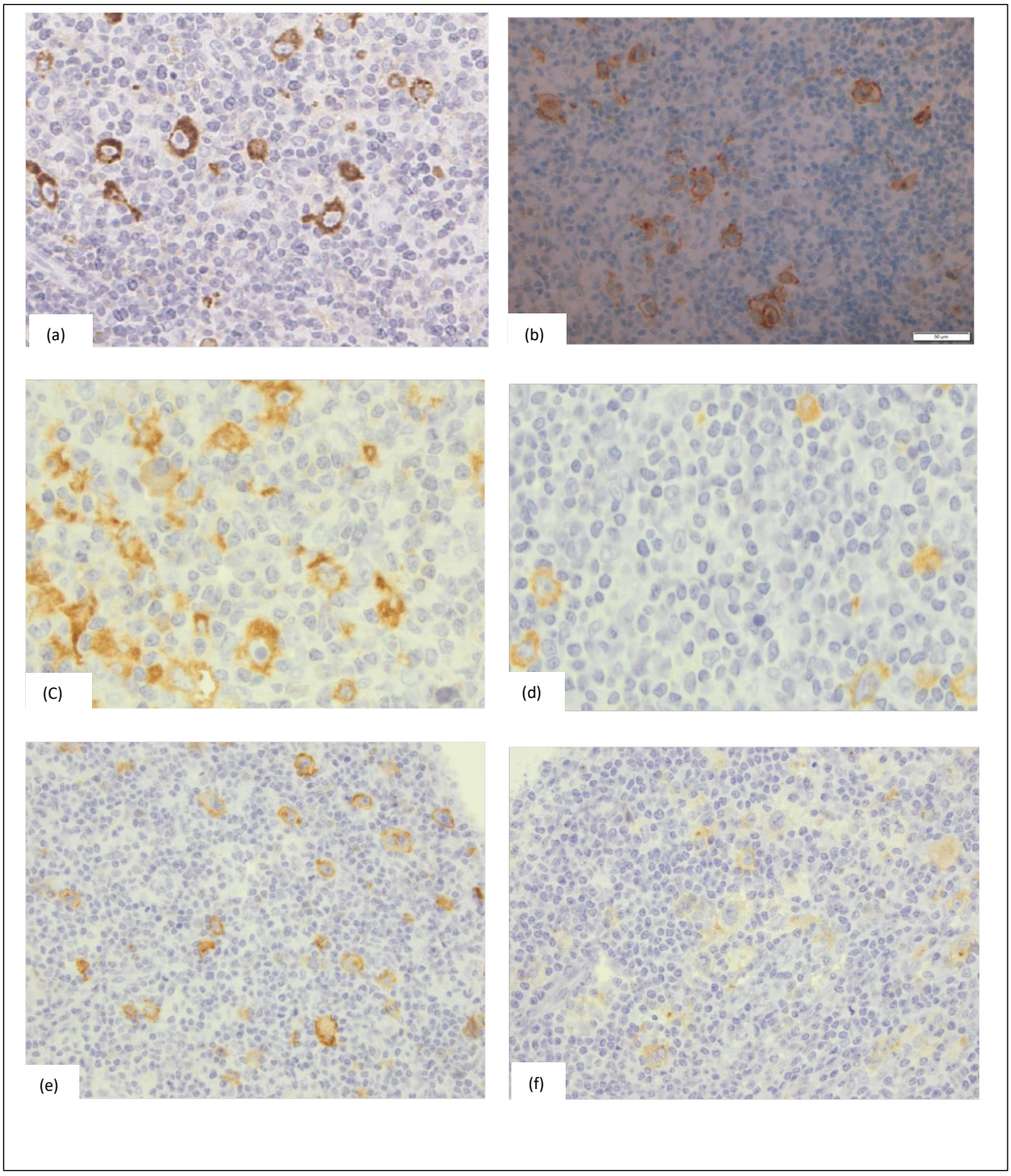


Figure 8. Staining intensity of IMP3 and CD30 markers on the HRS cells of HL cases, diagnosed between the years 2014 and 2019 at TASH. (a) Strong IMP3 staining. (b) Strong CD30 staining. (c) Moderate IMP3 staining. (d) Moderate CD30 staining. (e) Weak IMP3 staining. (f) Weak CD30 staining

Table 22. Ordinal regression prediction for the association between clinical characteristics and the staining intensity of IMP3 and CD30 biomarkers in HL cases, diagnosed between the years 2014 and 2019 at TASH

Markers	**Characteristics**	***Wald x²***	***OR (95% CI) P values***
n = 126			
IMP3 intensity			
	HL classes (CHL & NLPHL)	3.992	3.4 (.023-2.4) 0.046
	EBV+	4.919	2.3 (1.1-4.8) 0.027
	Sex	2.608	2.03 (0.9-4.8) 0.10
	Age	1.793	0.98 (0.96-1.007) 0.18
	HL subtypes	1.673	0.74 (0.5-1.2) 0.19
CD30 intensity			
	EBV+	14.512	4.25 (2.032-8.875) <0.001
	Sex	0.908	1.49 (0.65-3.4) 0.3
	Age	1.148	0.98 (0.96-1.01) 0.28

3.4.3. Association of the percentage of IMP3 and CD30 expression on HRS cells/each HL case with different clinico-pathological features

The mean percentage of HL cells expressing CD30 protein was compared with the mean percentage of HL cells expressing IMP3. Accordingly, the mean percentage of tumor cells expressing IMP3 was significantly higher, 78.8%, compared to CD30 64.9%, with *P* value <0.001 and 95% CI (9.53-18.29) Table 23. As demonstrated in Supplementary Table 24, there were no differences detected between the proportion of IMP3 and CD30 expression and sex or HIV status. The proportion of HRS cells expressing CD30 was higher in the cases with EBV

compared to EBV-negative HL (mean of 69.86 vs 57.18, respectively); with OR: 2.8, 95% CI 3.8 to 21.5, (*P* =0.005). Furthermore, a linear logistic regression was conducted for variables with *P* value = 0.25 (Table 24) entered in the model. The high proportion of tumor cells expressing CD30 was independently associated with the HL subtype. The proportion of CD30 positive tumor cells was 4.3 (19-51) times higher in CHL compared to NLPHL cases, *P*<0.001. The difference between EBV-related and EBV-unrelated cases was not significant in the linear regression model, (1.5 (-2-16), 0.14). There was no association between the proportion of HRS cells expressing IMP3 and the expression of LMP1.

Table 23. Details about the percentage of CD30 and IMP3 positive HRS and LP cells of HL cases, diagnosed between the years 2014 and 2019 at TASH

Immune marker	**Mean**	***P values***	**Std Error of mean**	**Median**	**Std. deviation of**	**Range**	**Percentile**		
N = 126							**25**	**50**	**75**
CD30%	64.9	<0.001	2.24	70	25.1	0-95	51	70	85
IMP3%	78.8		1.33	82	14.9	20-100	75	82	90

Table 24. Association of the percentage of IMP3 and CD30 expression with clinco-pathological features of 126 HL cases, diagnosed between the years 2014 and 2019 at TASH

Markers%, Characteristics	**Mean (Std error)**	**COR (95% CI), *P values***	**OR (95% CI), *P* value**
IMP3 %			
LMP1		1.3 (-1.8-8.9), 0.18	0.7 (-3-7), 0.4*
Positive (77)	80 (1.5)		0.9 (-3-8), 0.3**
Negative (49)	76 (2.5)		
Sex		0.2 (-5-7), 0.8	
Male (95)	78 (1.5)		
Female (31)	79 (2.7)		
HIV		0.8 (-8 -21.), 0.3	
Positive (4)	84 (7.5)		
Negative (122)	78 (1.4)		
Age		-0.7 (-10-5), 0.5	
≤45 (109)	79 (1.4)		

>45 (17)	76 (3.6)		
HL classes		1.8 (-1-18) 0.07	1.4 (-3-17), 0.17*
CHL (n= 116)	79 (1.3)		
NLPHL (n= 10)	70 (6.8)		
HL subtypes		1.2 (-1.2-5.6), 0.2	0.8 (-2-4), 0.4**
MCCHL (62)	80 (1.6)		
NSCHL (50)	77 (2.2)		
LDCHL & LRCHL (4)	83 (5.2)		
NLPHL (10)	70 (6.8)		
CD30%			
LMP1		2.8 (3.8-21.5), 0.005	1.5 (-2.-16), 0.14♦
Positive (77)	69 (2.6)		1.14 (-3-14), 0.25♦♦
Negative (49)	57 (3.9)		
Sex		0.6 (-7.4-13.2), 0.6	
Male (95)	65 (2.6)		
Female (31)	62 (4.5)		
HIV		1.08 (-11.5-38.9), 0.28	
Positive (4)	78 (12.5)		
Negative (122)	64 (2.3)		
Age		-1.5 (-22.6-3), 0.14	-1.2 (-19-4), 0.2♦
≤45 (109)	66 (2.4)		-0.3 (-14-10), 0.7♦♦
>45 (17)	56 (6.1)		
HL classes			
CHL (n= 116)	68 (2.1)	5.3 (24.5-54.5), <0.001	4.3 (19-51), <0.001♦♦
NLPHL (n= 10)	28 (7.4)		
HL subtypes		3.6 (4-15) <0.001	2.8 (2-14), 0.006♦
MCCHL (62)	70 (2.7)		
NSCHL (50)	66 (3.4)		
LDCHL & LRCHL (4)	58 (17.3)		
NLPHL (10)	28(7.4)		

*Linear regression, IMP3% dependent variable and LMP1 and HL classes the covariates. ** Linear regression, IMP3% dependent variable and LMP1 and HL subtypes the covariates. ♦Linear regression model, CD30% dependent variable and LMP1, Age and HL subtypes the covariates ♦♦ Linear regression model, CD30% dependent variable and LMP1, Age and HL classes the covariates

3.5. Prognosis and Overall survival of HL cases

3.5.1. The international prognostic score and treatment outcome (4-year OS)

The 4-year overall survival (OS) for HL (both the CHL and NLPHL) patients (n=71) in this study was 83.1% (Figure 9 a), with a 19-month median follow-up time. The 4-year OS of patients

with early and advanced stage disease were 100% and 78.2%, respectively. Patients presenting with stage I, II, III and IV had 4-year OS rates of 94.1%, 76.3%, 75.8% and 66.7% respectively (Table 25). Among patients with advanced stage disease, the 4-year OS among the high-risk group of patients (IPS≥ 3) was numerically lower than in the low-risk group of patients (IPS≤ 2), 68.9% and 93.3% respectively, but there was no statistically significant difference between the two groups (*P*= 0.177) (Figure 9 b).

As depicted in Table 25, LDCHL, the most aggressive HL subtype, was associated with a 3-year OS rate of 50%, while MCCHL and NSCHL was associated with 4-year OS rates of 86.5% and 89.9% respectively. Patients with the NLPHL subtype exhibited a 100% 4-year survival rate. Patients with lymphocyte count of ≤0.6x10^9/L had an inferior 4-year OS rate (70.8%) and this was the only prognostic factor with a significant impact on OS. Patients treated with combined chemotherapy/radiotherapy exhibited excellent survival, with a 4-year OS of 100%, whereas for patients receiving ABVD only, the 4-year OS was 77.9%. Only 5 HL patients with HIV were included in the survival analysis, all were alive up to the last date of follow-up data collection.

Table 25 4-year overall survival and prognostic factors of HL cases included in this study, diagnosed between 2014 and 2019 at TSH

Characteristics	**1-year OS%**	**2-years OS%**	**3-years OS%**	**4-years OS%**	***P value***
Overall survival	97.0	90.0	83.1	83.1	
IPS					0.177
IPS ≤ 2	100	93.3	93.3	93.3	
IPS ≥ 3	96.3	86.2	68.9	68.9	
Sex					0.746
Male	97.4	91.2	80.5	80.5	

Female	96.0	87.3	87.3	87.3	
Age group					0.4
0-9	91.7	91.7	91.7	91.7	
10-19	94.1	83.7	83.7	83.7	
20-29	92.9	74.3	49.5	49.5	
>30	100	90	90	90	
An Arbor staging					0.654
Stage I	94.1	94.1	94.1	94.1	
Stage II	94.1	76.3	76.3	76.3	
Stage III	-	90.9	75.8	75.8	
Stage IV	-	-	66.7	66.7	
Early and advanced stages					
(Stage I &IIA) early stage				100	0.140
Advanced stage (Stage I & IIB, III, IV)	96.1	87.4	78.2	78.2	
HL subtypes					0.721
MCCHL	96.2	86.5	86.5	86.5	
NSCHL	96.3	89.9	89.9	89.9	
LDCHL	100	50	-	-	
LRCHL	-	-	-	-	
NLPHL	100	-	-	-	
WBC count					0.069
WBC >10.04 x10^3/µl				100	
WBC≤10.04 x10^3/µl	95.4	92.6	85.3	76..3	
Lymphocyte x10^3/µl					0.015
>600	100	100	100	100	
≤600	94.3	82.6	70.8	70.8	
Monocyte x10^3/µl					0.078
> 0.24	100	94.7	88.8	88.8	
<0.24	91.8	82.8	74.5	74.5	
Hemoglobin g/dl					0.772
≥10.5	100	91.7	91.7	91.7	

<10.5	97.7	91.4	83.1	83.1	
Treatment					0.112
ABVD only				77.9	
ABVD plus[¶]				100	

ABVD plus[¶]: Patients treated with combined chemotherapy ABVD (doxorubicin, bleomycin, vinblastine and dacarbazine), COPDAC (cyclophosphamide, vincristine sulfate, prednisone, and dacarbazine)/Radiotherapy

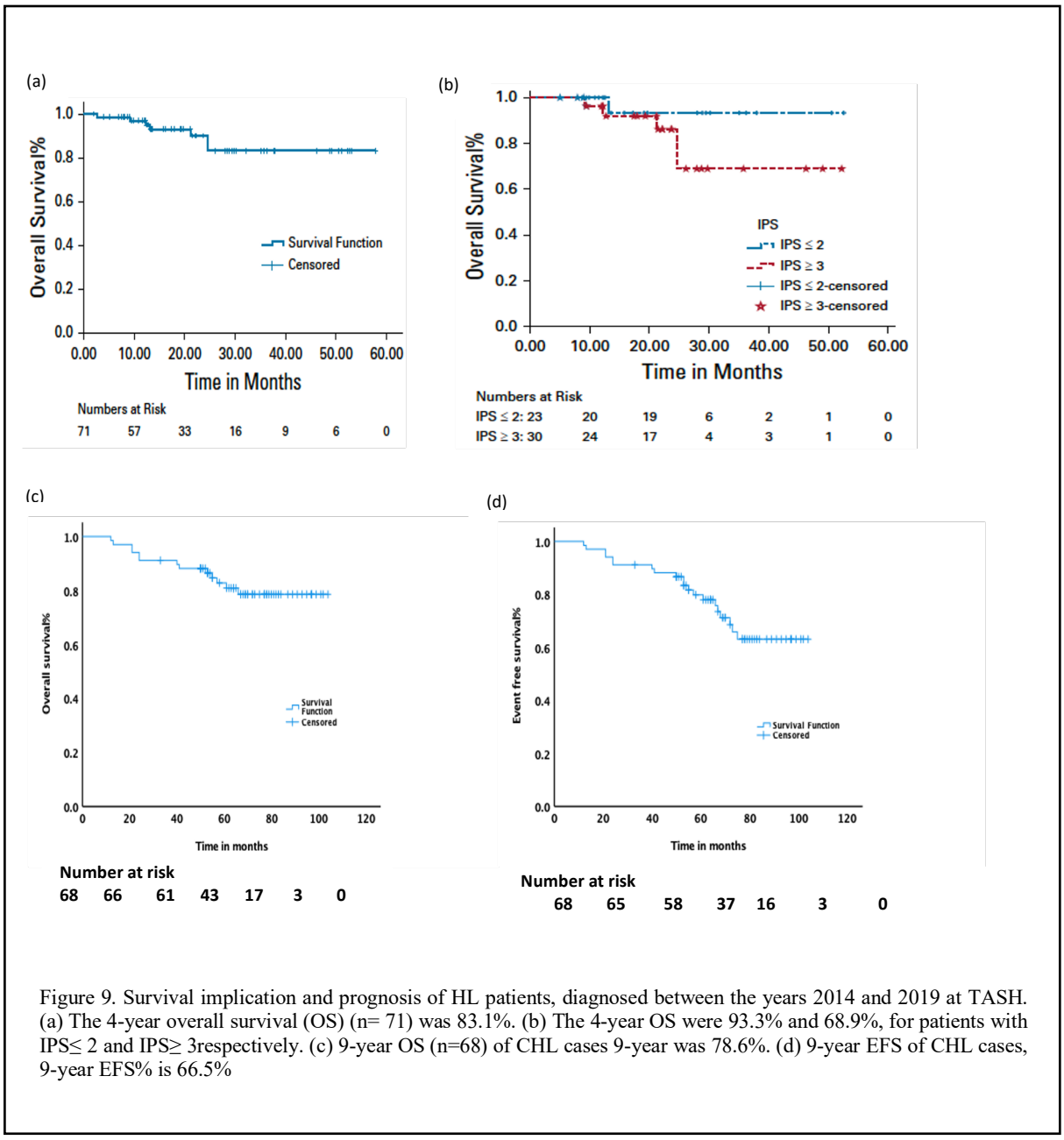


Figure 9. Survival implication and prognosis of HL patients, diagnosed between the years 2014 and 2019 at TASH. (a) The 4-year overall survival (OS) (n= 71) was 83.1%. (b) The 4-year OS were 93.3% and 68.9%, for patients with IPS≤ 2 and IPS≥ 3respectively. (c) 9-year OS (n=68) of CHL cases 9-year was 78.6%. (d) 9-year EFS of CHL cases, 9-year EFS% is 66.5%

3.5.2. 9-year survival according to clinical prognostic factors

The 9-year OS and EFS outcome of CHL cases (n=68) was 78.6% and 66.5% (Figure 9 c & d), respectively, with median follow-up time of 5.5 years (range 1-9 years). Cases in the IPS high-risk group had inferior OS and EFS compared to the group of cases in the low-risk group (Figure 10 e and f), with P value of =0.04, and OS% and EFS% of 63.5% and 45.5% respectively. Survival outcome remained significant for low lymphocyte count (Figure 10 c and d) with 9-year OS% and EFS% of 64.7% and 48.5%, *P*= 0.004 and 0.001, respectively. In univariate Cox-regression, the hazard ratio and 95% CI for OS and EFS for the group of cases with low lymphocyte count were (HR= 10.9, 95% CI: 1.4-84) and (HR= 5.4, 95% CI: 1.7-20), respectively. Patients with advanced stage had inferior EFS (Figure 10 b) with EFS% of 58.9%, *P* value of 0.03 compared to the group of patients in the early stage of the disease. The cases with advanced stage had lower overall survival outcome, OS% of 76.6, however, the difference was not statistically significant (Figure 10 a).

Lymphocyte depleted (LDCHL) 3 cases and one case of lymphocyte rich (LRCHL) had low OS and EFS outcomes (Table 26). However, the number of LDCHL and LRCHL cases was not comparable to the number of MCCHL and NSCHL cases (Table 26). CHL cases with HIV (4 cases) had inferior EFS (Table 26) EFS% of 50% and *P* value of 0.0016, compared to the CHL cases without HIV. Although, the CHL cases with HIV had low OS% compared to the OS% of cases without HIV, 37.5% and 81.4%, respectively. The difference between HIV-related and HIV-unrelated cases was not significant. Age, sex, and hematological parameters such as white blood cells and hemoglobin were not related to outcome (Table 26).

Table 26. Overall survival (9-year OS) and event free survival of CHL (Kaplan Mayer's test) (n=68) cases, diagnosed between the years 2014 and 2019 at TASH

characteristics	**9-year OS%**	***P* value**	**9-year EFS%**	***P* value**
9-year OS/EFS	78.6		66.5	
IPS		**0.04**		**0.04**
IPS<3	94.1		81.9	
IPS≥3	63.5		45.4	
Age		0.3		0.5
≤45	82.2		70	
>45	62.5		50.8	
Sex		0.8		0.9
Male	79.6		66.7	
Female	74.7		64.4	
Histopathological subtypes of cHL		**0.015**		**0.017**
MCCHL	78		69.8	
NSCHL	84		67.5	
LDCHL	66.7		33.3	
LRCHL	00		00	
Stage of the disease		0.06		0.06
Stage I	94.1		88.2	
Stage II	87.7		70.3	
Stage III	70.7		64.2	
Stage IV	53.8		29.7	
Early and advanced stage		0.09		**0.03**
Early stage	93.3		87.7	
Advanced stage	76.6		58.7	
WBC count		0.1		0.7
$<15x10^9$cells/L	74.6		65.7	
$\geq15x10^9$ cells/L	93.3		68.6	
Lymphocyte count		0.004		**0.001**
$\leq0.6x10^9$ cells/L	64.7		48.5	
$>0.6x10^9$ cells/L	95.7		88.5	
Hemoglobin level		0.1		0.2

≤10.5 g/dL	73.5		60.8	
>10.5 g/dL	94.1		81.9	
HIV status		0.1		**0.016**
HIV positive	37.5		50	
HIV negative	81.4		70.3	
EBV status		0.4		0.8
EBV positive	81		66	
EBV negative	72.4		66.4	
FoxP3		**0.015**		0.07
<9%	90.6		78.5	
≥9%	64.6		54.1	
PD1		**0.027**		0.3
<24.6	84.3		70.4	
≥24.6	57.1		57.1	

3.5.3. Survival implications of FoxP3 and PD1 expression

FoxP3+ cells and PD1+ cells in the tumor microenvironment (TME) of CHL (percent of positive cells out of the total cells in the TME) were treated as continuous data. Different cut-points were examined, but 50 (9%) and 60 (10%) percentiles of FoxP3, and 80 (24.6) percentiles of PD1were associated with significant overall survival (OS) impact in Kaplan-Meier analysis as depicted in Table 27.

Table 27. Different cut-offs, OS and EFS of FoxP3+ and PD1+ cells in the TME of CHL, diagnosed between the years 2014 and 2019 at TASH

Cut-offs (Percentile)	**Overall survival**	**Event free survival**
	HR (CI 95%), *P* value	**HR (CI 95%), *P* value**
FoxP3		
2 (10%)	1.7 (0.22-13.1), 0.6	1.07 (0.14-8.06), 0.9
3 (20%)	1.5 (0.34-6.9), 0.5	1.09 (0.26-4.7), 0.9
4 (25%)	1.3 (0.3-5.9), 0.7	1.02 (0.34-3.08), 0.9
5 (30%)	2 (0.5-9.3), 0.3	1.5 (0.5-4.5), 0.5
7 (40%)	2 (0.6-7.7), 0.3	0.9 (0.4-2.3), 0.9
9 (50%)	4.3 (1.2-15.7), **0.015**	2.4 (0.96-6), 0.052
10 (60%)	3.2 (1.06-9.4), **0.03**	1.6 (0.6-4), 0.3
14 (70%)	1.4 (0.5-4.3), 0.5	1.03 (0.4-2.6), 0.9
17 (75%)	1.6 (0.5-5.2), 0.4	0.9 (0.3-2.7), 0.8
18 (80%)	1.4 (0.4-5.1), 0.6	0.8 (0.2-2.9), 0.8
23 (90%)	2.6 (0.6-12), 0.2	2.02 (0.5-8.9), 0.3
PD1		
0.8 (10%)	1.06 (0.1-8.2), 0.9	1.5 (0.2-11.3), 0.7
1.6 (20%)	2.3 (0.3-18), 0.4	3.2 (0.4-23.8), 0.3
2.2 (25%)	3.3 (0.4-25.9), 0.2	2.2 (0.5-9), 0.3
3.3 (30%)	0.9 (0.3-3), 0.9	1.06 (0.4-3), 0.9
4.6 (40%)	0.6 (0.2-1.9), 0.4	0.9 (0.3-2.3), 0.8
5.8 (50%)	0.7 (0.2-2), 0.5	0.9 (0.3-2.2), 0.7
8.8 (60%)	1.03 (0.3-3.2), 0.9	1.3 (0.5-3.3), 0.5
18.5 (70%)	1.7 (0.5-5.4), 0.4	1.1 (0.4-3), 0.8
19.9 (75%)	2.2 (0.7-6.9), 0.2	1.08 (0.4-3), 0.9
24.6 (80%)	3.4 (1.1-10.6), **0.02**	1.7 (0.6-4.7), 0.3
48.9 (90%)	2.3 (0.5-10.5), 0.3	1.2 (0.3-5.6), 0.7

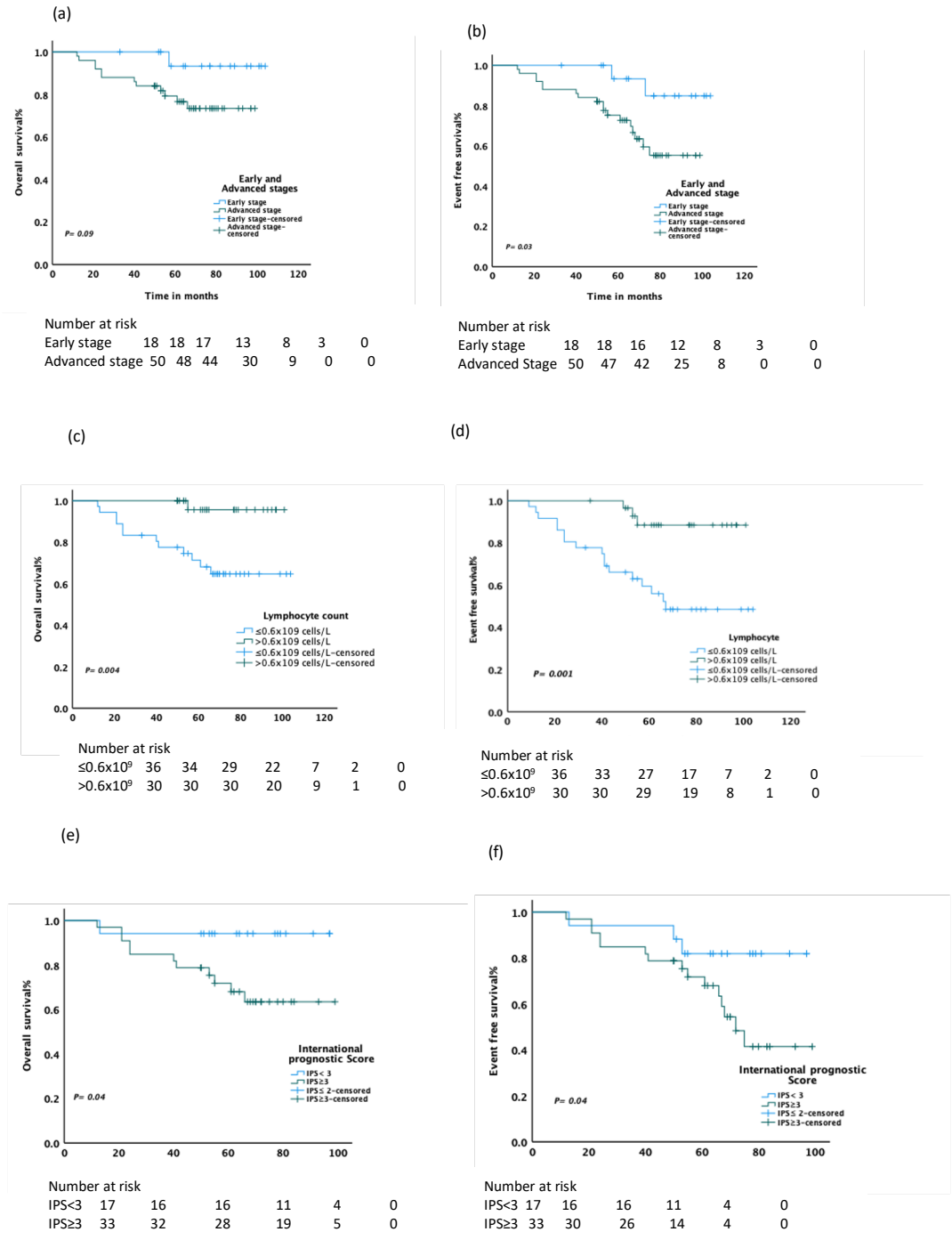


Figure 10. 9-year OS and EFS for the clinical characteristics of CHL, diagnosed between 2014 and 2019 at TASH. (a) The 9-year OS of cases in the early and advanced stage groups was 93.3% and 76.6%, respectively, P= 0.09. (b) 9-year EFS of cases in the early and advanced stage groups was 87.7% and 58.7% respectively, P= 0.03. (c) Lymphocyte at cutoff $0.6x10^9$ cells/L, the OS for the groups of cases with low ($\leq 0.6x10^9$ cells/L) and high ($>0.6x10^9$ cells/L) lymphocyte count was 64.7% and 95.7% respectively, P= 0.004. (d) Lymphocyte at cutoff $0.6x10^9$ cells/L, the EFS for the group of cases with low ($\leq 0.6x10^9$ cells/L) and high ($>0.6x10^9$ cells/L) lymphocyte counts was 48.5% and 88.5% respectively, P= 0.002. (e) The low-risk and high-risk groups of the IPS 9-year OS% for IPS<3 and IPS≥3 were 94.1% and 63.5%, respectively, P<0.04. (f) Low-risk and high-risk groups of the IPS, 9-year EFS% for IPS<3 and IPS>3 was 81.9% and 45.4%, respectively, P<0.04.

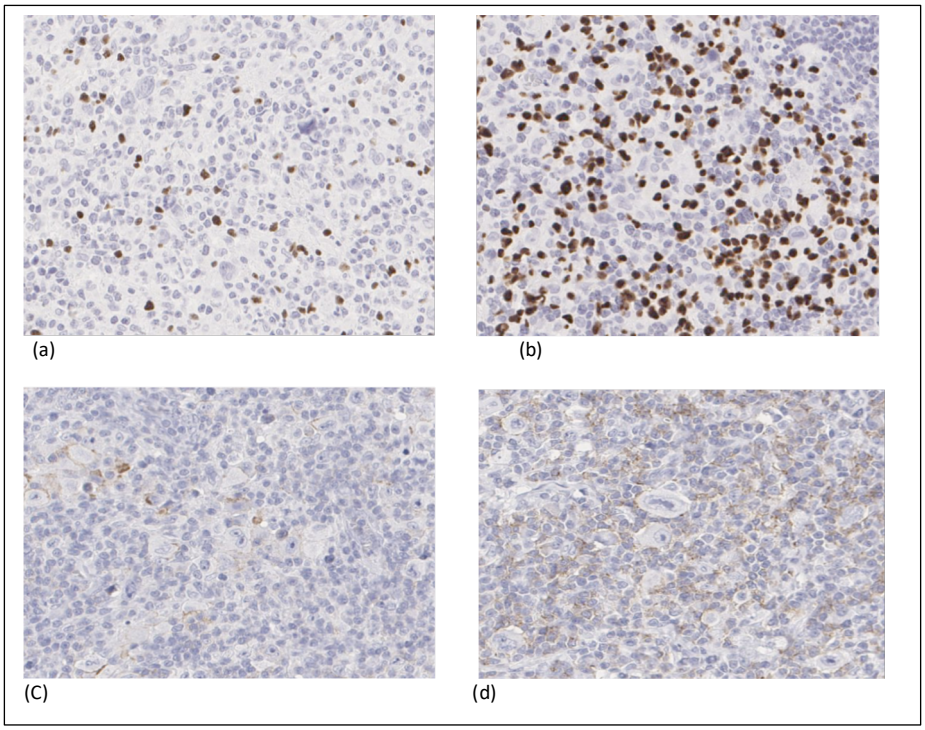


Figure 11. Immunohistochemical staining for FoxP3 and PD1 expression on the cells of tumor microenvironment of CHL at x20 magnification, diagnosed between 2014 and 2019 at TASH. (a) Low expression of FoxP3, (b) high expression of FoxP3. (c) Low expression of PD1, (d) high expression of PD1

High FoxP3 expression ≥9% (Figure 11 b) on the cells of TME (n= 31/67) compared to patients with low FoxP3 expression <9% (Figure 11 a) was associated with inferior 9-year OS and EFS in Kaplan-Meier test, OS% of 64.6%, *P*= 0.015 (Figure 12 a) and EFS% of 54.1%, *P*= 0.07 (Figure 12 b). Similarly, in univariate Cox-regression, the OS hazard ratio (HR) and 95% CI of the group of patients with high FoxP3 was HR= 4.3 and 95% CI= (1.2-15.7).

Similarly, high number of PD1+ cells (Figure 10 d) ≥ 24.6% in the TME of CHL (n= 12/63) was associated with inferior 9-year OS compared to patients with low PD1+ cells (Figure 11 c) <24.6 (n= 51/63), in Kaplan-meier test the OS% for high PD1+ was 57.1%, *P*= 0.02 (Figure 12 c). In Univariate Cox-regression HR and 95% CI for high PD1+ expression was HR= 3.4 and 95% CI = (1.07-10.6). In multivariate Cox-regression, survival outcomes remained statistically significant for the group of patients with lymphopenia (Table 28), HR and 95% CI for OS and EFS were (HR= 14, 95% CI: 1.6-121) and (HR= 5, 95% CI: 1.4-17), respectively. Both high FoxP3

and high PD1 expression remained statistically significant for overall survival in multivariate Cox-regression (Table 13), (HR= 8.6, 95% CI: 1.6-45) and (HR= 10.5, 95% CI: 2.6-42) for FoxP3 and PD1 expression, respectively.

Table 28. Multivariate Cox-regression survival analysis of CHL cases for variables with P value <0.1 in the univariate Cox-regression analysis, cases diagnosed between 2014 and 2019 at TASH

Variables	Overall survival HR (95% CI), *P* value	Event free survival HR (95% CI), *P* value
Lymphocyte/L ($\leq 0.6x10^9/>0.6x10^{9)}$	14 (1.6-121), 0.016	5 (1.4-17), 0.01
FoxP3+% (<9/≥9)	8.6 (1.6-45), 0.011	2 (0.8-5), 0.1
PD1+% (<24.6/≥24.6)	10.5 (2.6-42), 0.001	-
Early and advanced stage	6.8 (0.8-60), 0.08	2.8 (0.6-12), 0.1

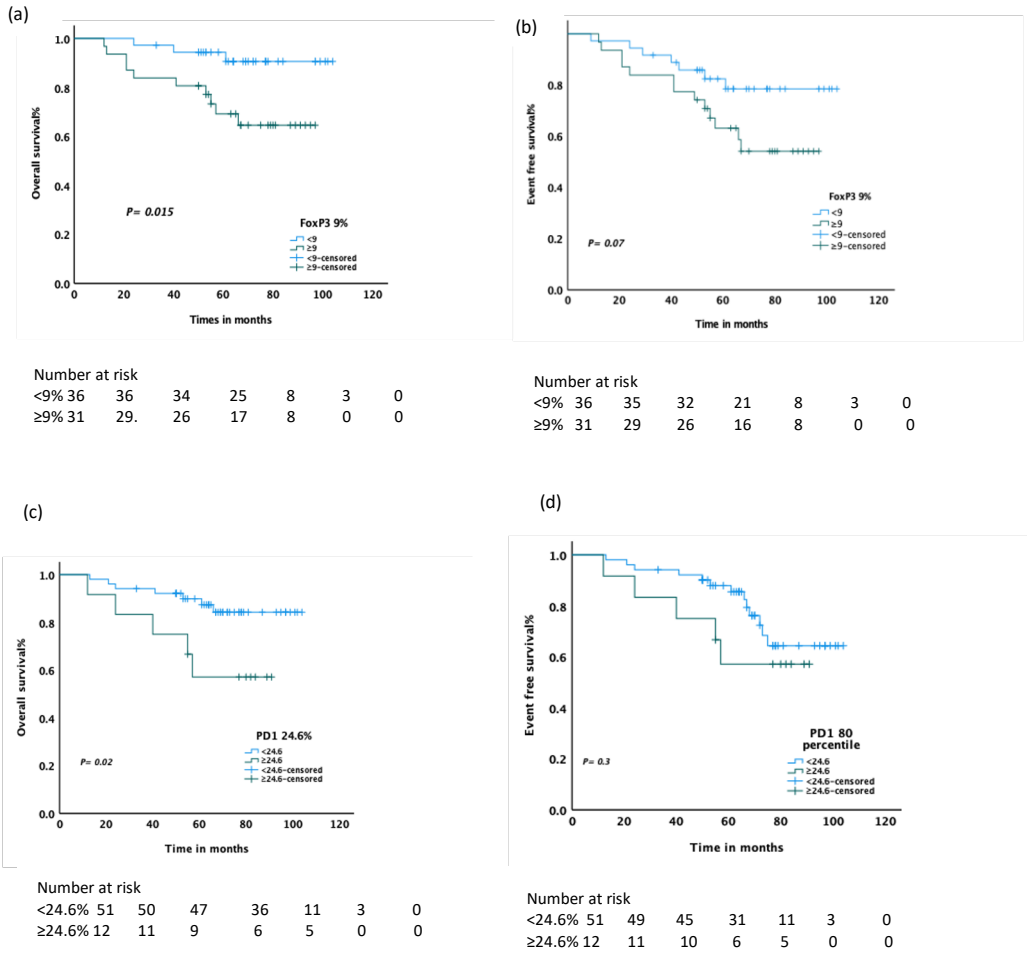


Figure 12. 9-year OS and EFS of CHL cases for FoxP3 and PD1 expression on the cells of TME, cases diagnosed between 2014 and 2019 at TASH. (a) OS% of FoxP3 at cutoff point 50 percentile (9%). The 9-year OS% of the group of cases expressed low FoxP3 (<9%) was 90.6% and was 64.6% for the group of cases with high FoxP3 (≥9%), P= 0.015. (b) EFS% of CHL cases for FoxP3 expression on the cells of TME at cutoff point 50 percentile (9%). The 9-year EFS% of the group of cases expressed low FoxP3 (<9%) was 78.5% and was 54.1% for the group of cases with high FoxP3 (≥9%), P= 0.07. (c) OS% of CHL cases for PD1 expression on the cells of TME at cutoff 80 percentile (24.6%). The 9-year OS% of the group of cases that expressed low PD1 (<24.6%) was 84.3% and for the group of cases with high PD1 expression (≥24.6%) was 57.1%, P= 0.02. (d) EFS% of CHL cases for PD1 expression on the cells of TME at cutoff point 80 percentile (24.6%). The 9-year OS% of the group of cases that expressed low PD1 (<24.6%) was 70.4% and for the group of cases with high PD1 expression (≥24.6%) was 57.1%, P= 0.3

4. Discussion

Hodgkin lymphoma shows differences in age distribution and proportions of histopathological subtypes in different geographical, genetic, and environmental settings (Saarinen et al., 2013). In high-income countries, HL shows a bimodal age distribution at diagnosis (Cozen et al., 1992, Thomas et al., 2002). In contrast, the present study showed more cases among the younger age group and was rare among the elderly. This is partly explained by the difference in demographic distribution, which is characterized by large proportion of younger age group and consequently, HL significantly decreased after the age of 50. This finding is similar to what has been reported from previous studies (Riyat, 1992, Olu-Eddo and Omoti, 2011, Tefera et al., 2016, Zeggai et al., 2016)

Several risk factors have been related to HL such as sex, age, race, genetic and environmental factors (Cozen et al., 1992, Thomas et al., 2002). Male sex has been stated as a risk factor for contracting HL (Cartwright et al., 2002). Moreover, the incidence of HL was found to be higher in males more than in females of all races (Shenoy et al., 2011). Similarly, in this study, the incidence of HL was higher in males compared to females. However, the male to female proportion of HL cases in Ethiopia was relatively higher than in high-income countries (Motawy and Omar, 1986, Cozen et al., 1992); and was lower than what has been reported from other Sub-Saharan African countries (Riyat, 1992, Olu-Eddo and Omoti, 2011).

In addition, HIV has been suggested as a risk factor for developing Hodgkin lymphoma (Goedert et al., 1998). A recent study assessed the impact of HIV epidemic on the increasing incidence of cancer in Sub-Saharan Africa and reported 8.6% of cases to be associated with

HL (Menon et al., 2017). Likewise, the present study has detected a relatively high proportion of HIV associated HL (13%), 3 folds higher than that reported from the USA (Shiels et al., 2014).

The majority of HL cases in this study (65.8%) categorized as stage I and II at diagnosis is in contrast to a study conducted in Nigeria,which reported a predominance of late stages (Olu-Eddo and Omoti, 2011). Furthermore, this finding showed that 63.7% of patients with HIV-associated HL presented with early stages at diagnosis, which is consistent with a study that reported HIV patients to be more likely to present at early stages of any type of cancer compared to the general population in Africa (Menon et al., 2017).

In HL, the hemoglobin level and peripheral white blood cell count, and their composition are important prognostic factors at diagnosis and at treatment evaluation and are part of the IPS score for patients with advanced-stage disease (Cheson et al., 2014). In this study, 65.1% of HL cases presented with low hemoglobin and leukopenia was also present in the majority of cases. HIV-related HL cases presented with monocytopenia, due to that monocyte expresses CD4 receptor and CCR5 co-receptors and can be infected with HIV. Similarly, 53% of HL patients were found to be anemic in a study conducted in Pakistan (Yasmeen et al., 2019).

The dominant HL subtype in this study was MCCHL (50.4%), higher than findings from Kenya (44.9%) (Riyat, 1992) and lower than what was reported from Nigeria (64.3%) (Olu-Eddo and Omoti, 2011). However, the second dominant subtype was NSCHL in the present study, which is different from the findings from Kenya and Nigeria, in which NLPHL was the second dominant subtype (Riyat, 1992, Olu-Eddo and Omoti, 2011). In contrast, a recent study from Egypt reported NSCHL as a common subtype of HL (Zeggai et al., 2016). Studies from the USA and Europe have indicated the NSCHL subtype to be the dominant subtype (Rapezzi et al.,

2001, Shenoy et al., 2011). The Kenyan and Nigerian discrepancy might be due to differences in environmental factors and exposure to infections (Belkaid et al., 1995, Glaser et al., 1997). Most HIV-positive HL cases were MCCHL subtypes followed by NSCHL. Our finding is similar to the report of a study from South Africa (Patel et al., 2011). Several reports suggest the possible association of MCCHL subtype of HL with viral infection, especially of Epstein Barr Virus (EBV) (Leoncini L et al., 1996, Glaser et al., 1997).

Immunohistochemical phenotyping has contributed new insights into the understanding of the nature, the diagnosis, and the classification of Hodgkin lymphoma. However, some HRS cells lack typical immunohistochemical (IHC) characteristics (Karnik et al., 2003) and may not be covered by the panel of markers suggested by WHO to be used for differential diagnosis of HL CD30, CD15, CD20, PAX-5, CD79a, CD45, LMP1, OCT2, BOB1, CD3, CD2, perforin/granzyme B, CD43, EMA, ALK-1 (Stein et al., 2017). The activation marker of lymphocytes, CD30, is also expressed in infectious mononucleosis (Reynolds et al., 1995), and CD30 and CD15 are consistently expressed in anaplastic large T-cell lymphomas (ALCL) (Ranuhardy et al., 2018). Moreover, some HRS cells may not express CD30. In this study, 4.2% and 17.5% of HL cases were found to be negative for CD30 and CD15, respectively, and 2.5% negative for both markers. All CD30 negative cases were of the NLPHL subtype and 40% and 50% of NLPHL cases were CD30 and CD15 negative, respectively, and 14.5% of the CHL subtype was CD15 negative.

Previous studies have indicated the probability of using IMP3 as a diagnostic (Shi et al., 2011, Wachter et al., 2012) and prognostic (Huang et al., 2017, Burdelski et al., 2018) biomarker in several malignancies, including HL. The expression of IMP3 in CHL in different studies were 64.3% (Zhang and Tsang, 2018), 85.7% (Masoud et al., 2019) and 98.6% (Tang et al., 2013) ,

and for NLPHL 92.3% (Zhang and Tsang, 2018), 50% (Masoud et al., 2019) , and 100% (Tang et al., 2013). In the present study, all cases of CHL and NLPHL expressed IMP3 protein (positive cytoplasmic staining). The expression of CD30, CD15, and IMP3 immunohistochemical staining of HL cases in the present study were 96.8%, 75.4%, and 100% respectively. Moreover, the percentage of HRS cells expressing CD30 protein/case was significantly lower when compared with the percentage of HRS cells expressing IMP3. The strongest expression of IMP3 was observed in MCCHL, followed by NSCHL, a finding that may warrant further investigation. The expression of the EBV-related marker, LMP1, was associated with high expression of both CD30 and IMP3. Studies have shown that viral infection may induce the expression of CD30 on B and T-lymphocytes (Froese et al., 1987, Amakawa et al., 1996). LMP1 induces the activation of several cellular pathways in the HRSc (Kieser et al., 1997, Dutton et al., 2005, Holtick et al., 2005), that may favour the aberrant expression of genes including IMP3.

The association of EBV with HL has been established. Although EBV seroprevalence was high in Sub-Saharan African countries, studies related to EBV association with HL are scarce. EBV positivity is highly age-dependent, with the highest fraction in younger HL patients and a preponderance among males. In this study, CHL cases in the group of children and young adults (age ≤24 years) were most often associated with EBV (80%). This finding is consistent with results from Kenya and the Middle East (Weinreb et al., 1996, Al-Salam et al., 2008) and in contradiction to what has been reported from Western countries (Enblad et al., 1999, Trimèche et al., 2007). In contrast to developed countries, EBV infection in developing countries is acquired during the early age of life (Ferressini Gerpe et al., 2020). The present

study showed that male HL cases were more often associated with EBV, confirming previous findings (Keresztes et al., 2006, Trimèche et al., 2007).

The sex variation in EBV infection between males and females may either be due to a higher rate of HL among males, as reported previously, or due to sex genetic variation (Cozen et al., 1992, Cartwright et al., 2002, Shenoy et al., 2011). Additionally, female hormones are reported to protect against EBV infection (Glaser et al., 1997). In the present series, the MCCHL subtype was more often associated with EBV infection, followed by NSCHL, while all NLPHL cases were negative for EBV. The expression of LMP1/EBER has been associated with different clinicopathological features of HL, such as sex, age, and HL subtypes (Trimèche et al., 2007).

EBV has been considered a risk factor for several types of lymphomas, including HL. However, the prevalence of EBV with HL has shown different association patterns in developing and developed countries. In the current study, EBV was detected in 61.1% of HL cases, in line with findings from other sub-Saharan countries (Chang et al., 2009) whereas, in the USA and Europe, 30%-50% of HL cases are associated with EBV . On the other hand, 60-100% of HL cases have been associated with EBV in Asian and Sub Saharan African countries (Kusuda et al., 1998). The prevalence and age variations in EBV infection between developed and developing countries are perhaps due to socioeconomic, hygienic, geographical and genetic differences (Chang et al., 1993, Glaser and Jarrett, 1996, Chang et al., 2009).

The expression of the biomarkers used in this study by corresponding immune cells have been confirmed and reported previously in several studies. For instance, the transcription factor C-maf has been used as an identification marker for Th2 cells in several series (Nurieva et al.,

2003, Atayar et al., 2007). The transcription factors FoxP3, T-bet and C-maf are predominantly expressed on T-reg, Th1 and Th2 cells in the TME of HL (Schreck et al., 2009), respectively. Although, T-bet and C-maf have been detected in the HRS-cells, their expression is very rare (Atayar et al., 2007, Schreck et al., 2009). In the TME of HL (CHL & NLPHL), the frequency of CD4+ cells was two times higher than that of CD8+ cells, this finding is in line with earlier reports (Henry et al., 2018, Menéndez et al., 2022). Daussy and coworkers described the CD4: CD8 ratio in lymphomas and reported that the CD4 cell population was higher than the CD8 cell population in HL, a result comparable to the present findings. In contrast, the CD4:CD8 ratio is inverted in diffuse large B-cell lymphoma (DLBCL) (Daussy et al., 2013).

In the TME of both types of HL (CHL and NLPHL), the expression of T-reg, Th1 and Th2 biomarkers (CD4+ cell subsets) showed significant variation. Most cells in the CD4+ population were found to be of Th2 subset, followed by Th1 and T-reg subsets. The abundance of C-maf+ cells in the TME could reflect the polarization of CD4+ cells to differentiate into the Th2 subset. The TME of CHL is reported to be high in C-maf+ and FoxP3+ cells (Schreck et al., 2009). The tumor cells of HL release signals to manage the type and activities of cells in their microenvironment, ensuring their proliferation, existence and escape immune surveillance (Schreck et al., 2009). T-reg transcription factor (FoxP3) expression was higher among CHL cases. In contrast, the number of PD1-expressing cells was higher among NLPHL patients. In addition, PD1 was higher in NLPHL when compared to the EBV-unrelated CHL. The finding of this study indicates that the quantity and type of CD4+ cell subsets in the TME of HL may vary depending on the presence of EBV infection. On the other hand, it has been reported that NLPHL microenvironment has high proportion of PD1 cells and low proportion of T-reg (Visser et al., 2016). Male sex was associated with higher expression of

FoxP3 expression, a finding similar to what has been reported previously (Gunduz et al., 2016).

The present study showed that the type and proportion of immune cells in the TME are highly influenced by the presence of EBV in the tumor cells (LMP1/EBER) of CHL cases. The abundance of CD4+ cells was similar in the microenvironment of EBV-related and EBV-negative CHL cases, as reported previously (Chetaille et al., 2009). In contrast, the CD8+ cell population was higher in the EBV-related HL group. In Foxp3 and T-bet, the transcription factors of T-reg and Th1 cells, were expressed at high levels in the microenvironment of EBV-related CHL. The abundance of CD8 and T-bet, biomarkers of T cells related to the cellular-mediated immune response, was high in EBV-related CHL. However, it is possible that the concomitant recruitment of cytotoxic T cells and Th1 cells with T-reg cells in the TME of EBV-related HL may suppress the cytotoxic activities of CD8+ T cells and Th1 cells, thereby ensuring the persistence and proliferation of HRSCs. In line with this finding, other studies had indicated that HL with EBV was dominated by CD8+ T cells and a milieu rich in Th1 cytokines (Duffield et al., 2017, Wu et al., 2016). This indicates that EBV infection may have a role in altering the immune component of the microenvironment of HL.

The finding that while EBV-related CHL was rich in Foxp3+ cells and the TME of EBV-unrelated CHL was rich in PD1+ cells show the differential recruitment of T cells (Foxp3+ vs PD1+) with immune suppressive/regulatory activities. This shows the existence of a differential strategy of HRSCs to escape immune surveillance in the presence or absence of EBV. The difference in the cells expressing Foxp3 between the EBV-related and EBV-unrelated TME is significant, a result contradicting what has been reported previously (Wu et al., 2016). However, the association of T-reg cells with CHL, reported by Wu et al (2016), was also detected in the

present series of cases. Furthermore, the present finding regarding the recruitment of T-regs in high quantities in the TME of EBV-related CHL is consistent with reports from Brazil (Assis et al., 2012) and France (Morales et al., 2014) and contradicts a more recent reports from Germany (Duffield et al., 2017). However, no difference was reported by Duffield and coworkers in the abundance of PD1+ cells between EBV-related and unrelated HL (Duffield et al., 2017). The difference in the reported findings between the studies appears to be due to the method used for scoring the immune cell signature.

In this study, the tumor cells in CHL were surrounded by a diverse population of T-cells within the tumor microenvironment (TME), however, it was reported that these T-cells often fail to eliminate the malignant cells (Burdelski et al., 2018). In the EBV-related CHL cases, high number of Th1 and cytotoxic T-cell the TME were detected. Reports showed that EBV in CHL might be involved in the genetic dysfunction of tumor cells (Kieser and Sterz, 2015, Liao et al., 2020), which can induce the production of cytokines and chemokines used for the recruitment of various types of immune cells to the TME. These chemokines triggering the migration of Th1 and cytotoxic T-cells to the tumor microenvironment. However, due to the presence of regulatory T-cells the cell-mediated immunity might be suppressed in the TME of EBV-related CHL (Gandhi et al., 2006). Although the T-cell exhaustion marker, PD1, was infrequent in CHL despite the high expression of the programmed cell death ligand (PDL1) (Taylor et al., 2023), 95.5% of the CHL cases in this study expressed PDL1 in both tumor cells and the cells of TME irrespective of EBV status.

The international prognostic score was associated with adverse survival outcomes (OS and EFS) in the group of HL patients included in this study. That is, the 4-year and 9-year OS was estimated to be 83.1% and 78.6%, respectively. This is similar to that reported from Malawi

(83% 1-year OS rate) (Stanley et al., 2017) and Nigeria that reported a 84.3% OS rate (Egesie et al., 2018). A study from Turkey reported a 10-year OS of 84% (Kılıçkap et al., 2013), and others from the USA and the Nordic countries have reported a 5-year relative survival ratio of 80% (Storm et al., 2010, Shenoy et al., 2011).

In Ethiopia, the standard first line treatment regimen for HL is ABVD. However, the 4-year overall survival of patients who received ABVD only was 77.9%, a result which is similar to what was reported by Santoro and coworkers (Santoro et al., 1987) from Italy, but lower than reported from other populations (88-90%) (Moccia et al., 2012, Kılıçkap et al., 2013). In contrast, the group of HL patients that received combined chemotherapy/radiotherapy showed excellent outcomes in this study.

This study was able to confirm the prognostic impact of the IPS score, with the strongest prognostic factor being lymphocytopenia. Although it is widely held that MCCHL and LDCHL are considered to be the HL subtypes associated with the worst survival outcome and NLPHL subtype to be associated with a good prognosis and survival outcome (Bröckelmann et al., 2016). In this study, the prognostic assessment of histopathologic subtypes showed that LDCHL was associated with the most inferior outcome, followed by MCCHL and NSCHL, and all patients with NLPHL were long-term survivors. HIV-related CHL cases had inferior survival outcomes, this may be due to a weak immune system. Survival outcome of HIV-related cancer was inferior compared to that of HIV-unrelated cancer (Marcus et al., 2015).

Furthermore, a significant finding of this study was the association of high expression of PD1 and FoxP3 in the TME of CHL with adverse survival outcomes. The result remained significant for both immune markers after multivariate Cox-regression. The proportion of PD1+ and

FoxP3 cells with prognostic impacts was determined by using percentiles of positive cells for the biomarkers. This finding is consistent with previous reports concerning the adverse impact of high PD1 (Greaves et al., 2013, Hollander et al., 2017) and FoxP3 expression in the TME of CHL (Schreck et al., 2009). In contrast, Chetaille's group had reported low FoxP3 expression to be related with poor survival outcomes (Chetaille et al., 2009) indicating that the interaction between HRS cells and immune cells within the TME is complex and needs further investigation.

Knowledge about the association of the increased number of PD1+ cells, in the tumor microenvironment of HL with inferior treatment outcome has led to targeting the PD1 pathway as a powerful therapeutic strategy for treating Hodgkin lymphoma (Younes et al., 2016). Drugs known as immune checkpoint inhibitors, such as pembrolizumab and nivolumab, block the PD1 pathway and enhance the immune response against cancer cells. Although, the $CD4^+$ T cells and $CD8^+$ T cells are abundant in the tumor microenvironment of CHL (Bertuzzi et al., 2021), the antitumor activities of immune cell infiltrates are limited. It has also been shown that immune cells in the TME of CHL can exhibit senescence, or in some cases, promote the growth and proliferation of tumors (Schreck et al., 2009). Additionally, there is evidence that certain immune cells can be manipulated by the tumor to support their survival and expansion (Ansell, 2015, Menéndez et al., 2022). In CHL, the cellular mediated immune response may be hindered either by the suppressive activity of the regulatory T cells or due to the expression of, the exhaustion marker, the programmed cell death-1 (PD1) on effector T cells. The contribution of regulatory T cells to the induction of effector T cell senescence in the tumor microenvironment (Liu et al., 2018, Li et al., 2019) may explain the poor prognosis among the group of CHL with high FoxP3+ cells, observed in this study. Due to

the alternative immunosuppression mechanism of T-regs, developing new therapeutics targeting T-regs in the TME of CHL might be of interest. In the era of personalized medicine, immunotherapy has emerged as an important treatment strategy for malignancies, including Hodgkin Lymphoma (Ramos et al., 2020, Straus et al., 2021). The finding of this study pinpoints FoxP3+ cells as a potential target for immunotherapy in the TME of CHL.

5. Limitations of the study

The study used tissue microarrays (TMA), which enables the study of large samples and an image analysis platform to quantify the expression of biomarkers in the TME of HL. Although the high association of EBV with HL made it possible to compare the microenvironment of EBV-related and EBV-unrelated cases, since the HL cases were from a Sub-Saharan country, the study has the following limitations:

1. It is a single-center hospital-based retrospective study, and the sample size was relatively small, which may affect the strength of the findings.
2. As the study included only cases who were attended by or referred to TASH during the study period, bias issues concerning the number of patients who are treated at TASH compared to the patients who did not get access to diagnosis and treatment at TASH may be raised.
3. The lack of organized cancer and death registry system in Ethiopia, which may underestimate lymphoma related deaths.
4. Due to resource limitations, multiplex immunohistochemistry was not conducted.

6. Conclusions and recommendations

6.1. Conclusions

The population at risk for HL in Ethiopia are the adolescent and young adults. As such, the risk of HL declined gradually in older ages. The majority of HL cases in this study were from regions near Addis Ababa, and very few from more distant regions, indicating that most of the population of the country was not able to get access to cancer medical services, including HL cases.

For proper diagnosis and treatment of HL, IMP3 protein can be a novel candidate immune marker. It could be used for the differential diagnosis of HL and could augment the diagnostic efficiency of CD30 and CD15-based tests. IMP3 can be used as an additional marker in the diagnosis of HL due to its relatively high expression as compared to other markers and its ability to specifically differentiate HRS and LP cells against the complex background.

The prevalence of EBV infection was high among HL cases in Ethiopia, with males, children, young adults, and MCCHL significantly affected. The cellular composition of the microenvironment in HL is highly affected by EBV infection, and the type and abundance of cells in the TME of HL are determined by EBV infection. EBV-related and EBV-unrelated HL use FoxP3+ cells and PD-1+ cells, respectively, to escape immunosurveillance. The difference in the microenvironment between the two groups of CHL may have clinical implications. In addition, investigating and understanding the microenvironment of HL, in general and the microenvironment of EBV-related and EBV-unrelated CHL, in particular is of utmost importance in the growing era of immunotherapy. Therefore, the underlying mechanisms of

HL biology must be fully understood to bring molecular and pathology data into a clinical context for the best-individualized treatment of patients.

The study confirms the prognostic implications of IPS, PD1 and FoxP3 and their association with adverse survival outcomes. Overall, after appropriate diagnostic workup and standard chemotherapy, the treatment outcome for the patients, included in this study, was excellent and comparable to that of most advanced societies, in spite of the wide resource gap.

6.2. Recommendations

- A sustainable supply of reagents and antibodies to do immunohistochemistry, flow cytometry, and molecular tests must be made available in health facilities to provide quality and advanced services to cancer patients.
- Access to HL treatments like immunotherapy and clinical trials with novel agents, which are generally restricted to high-income countries, should also include low-income countries.
- Since the TME is the potential target for immunotherapy, extensive studies should be conducted to investigate the immune cells in the TME, and low-income countries must be part of ongoing clinical trials in immunotherapy.
- Research on tumor microenvironment should be conducted to uncover novel potential treatment targets.

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Appendices

Appendix 1: Supplementary Figure

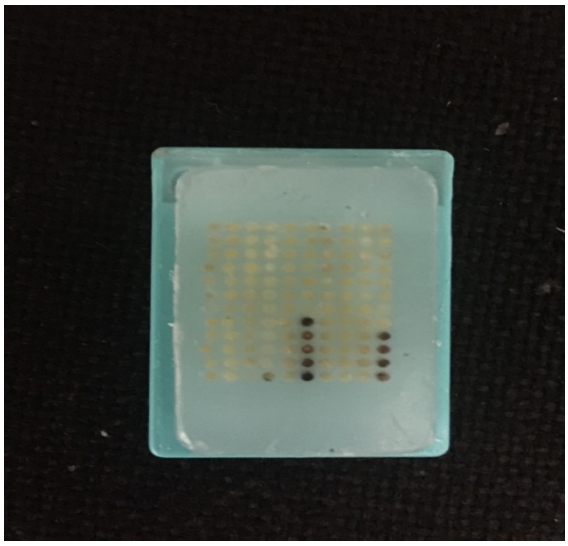

Fig. S1. Tissue microarray (TMA) block prepared from whole tissue blocks of the study participant, HL cases diagnosed between 2014 and 2019 atTASH

Appendix 2: IHC protocol optimization at Pathology Department

Immunostaining-protocol (IHC) Polymerase based IHC

- Procedure is done at room temperature except for microwaving steps.

1. Section preparation:
    - 3-4mm sections prepared on slides and let to be dried overnight
    - Dewaxed on oven at 60^0 C for 60 min.
    - Let the slide to be cool then either stain or store at 4^0 C

Staining procedure

1. Deparaffinize slides through two changes of xylene, 2x5mins.
2. Hydrate slides to water by dipping them absolute ethanol (99.5%) for 5 mins, 96% ethanol for 5 mins, and distilled water for 5 mins.
3. Place slides in microwave slide dish (plastic works best with a plastic slide rack) and cover with citrate buffer for PH 6 or EDTA solution or other buffer used for pretreatment.
4. Microwave on high power (800W) until boiling, ca 8 mins. Reduce the effect to 300 W and run for 15 mins. (Times may vary by microwave, but the important thing is to make sure the slides boil rapidly for 10 minutes minimum and do not dry out. An additional container of citrate buffer may be microwaved together with the slides to allow for refilling of the slide dishes.)
5. Cool for a minimum of 20-30 minutes. Cooling time is critical.
6. Rinse in tap water for 5 mins.
7. Place in wash buffer (PBS) for 5 mins.
8. Dry excess PBS from slides and circle tissue with hydrophobic pen leaving 1mm space between tissue and circle. Be sure that tissue never dries out.
9. Block endogenous peroxidase activity by covering circled area with solution from bottle #1 of DAKO kit and incubating for 5 minutes.
10. Rinse in PBS, X2 then remove or dry the excess buffer
11. Cover circled area with primary antibody or negative control solution and incubate for 30 minutes.

12. Rinse in PBS, X2 remove the excess buffer
13. Cover circled area with EnVision (HRP-conjugate polymers) bottle #2 from DAKO kit and incubate for 30 minutes.
14. Rinse in PBS, X2 dry the excess buffer
15. Prepare DAB according to number of slides using bottles #3a and #3b from DAKO kit. (1ml of bottle #3a to 1 drop of bottle 3b)
16. Cover circled area with prepared DAB substrate -chromogen solutin and incubate 10 minutes. Keeping this time constant allows for more accurate duplication of titers.
17. Rinse with distilled water, X2
18. Counterstain with hematoxylin for 4 mins.
19. Rinse with tap water, X3.
20. Dehydrate slides by 95% ethanol for 5mins followed absolute ethanol.
21. Clear slides by dipping twenty times in three changes of xylene or for 5 mins.
22. Coverslip slides with Protex or other xylene-compatible mounting medium.

Appendix 3: Solutions one could prepare if not available as ready in the lab

Solutions:

Citrate Buffer = 10um citrate buffer pH6.0

Method:

Measure 1 liter distilled water

Add 2.1gm of citrate acid monohydrate (mw=210.1)

Adjust pH to 6.0 with sodium hydroxide or HCl as necessary

Antibody dilutant = 1% albumin in PBS with 0.1% NaAzide

Method

Make stock 10% NaAzide

Measure 10ml distilled water

Add 1gm powdered NaAzide

Measure 100ml PBS working solution

Add 1gm powered albumin

Add 1 ml 10% stock NaAzide

25X PBS (phosphate buffer)

360g Sodium Chloride

66g Sodium phosphate (monobasic, monohydrate)

376g Potassium phosphate (dibasic)

add distilled water up to 2 liters. (pH should be around 7.3)

1X PBS (working solution)

dilute the 25X PBS 1:25 with H2O

Citrate Buffer, pH 6.0

2000 ml:

Citrate acid	4.2 g
Dest. water	1900 ml

Adjust pH to 6.0 with about 25ml 2M NaOH. Add dest. water to a total volume of 2000 ml.

Tris-EDTA Buffer, pH 9.0

2000 ml:

0.5 M EDTA	4 ml

1 M Tris	20 ml
Dest. water	1900 ml

Adjust pH to 9.0 with HCl (10%). Add dest. water to total volume of 2000 ml.

Appendix 4: **Demographic, clinical and risk factors data collection form for HL cases**

Name of person taking the information _____

Occupation _____

1. Demographic information

1.1. Respondent ID. _____ Tel. No. _____

Biopsy No. _____ Biopsy collection site _____

1.2. 1.2 Sex

☐ Male ☐ Female

1.3. 1.3 Age _____

1.4. 1.4 Marital status

☐ Single ☐ Married ☐ Divorced ☐ Widowed ☐ NA

1.5. Ethnicity _____

1.6. Permanent residence _____

1.7. Occupation

☐ Farmer ☐ Housewife ☐ Daily laborer ☐ Merchant

☐ Jobless ☐ Industry worker ☐ Government employee

1.8. Educational level

☐ Uneducated ☐ Elementary school ☐ Graduated from High school

☐ Higher education ☐ Other

1.9. Number of family member _____

1.10. Monthly income of the participant or the family of the participant

☐ Low ☐ Middle ☐ High

1.11. Region and town/village from where the case come from_____

2. Clinical Information

2.1. Presentation

A. Enlarged Lymph nodes: ☐ Yes ☐ No

B. Locality of the enlarged lymph node

☐ Cervical ☐ Axillary ☐ Supraclavicular ☐ Inguinal

C. Extranodal site

☐Mediastinal mass ☐ Spleen ☐ Liver

☐Bone ☐ Other (Indicate) _____

D. “B” Symptoms

Night sweats ☐Yes ☐No Fever ☐ Yes ☐ No

Weight loss ☐ Yes ☐ No

E. Other Symptoms

Pruritis ☐ Yes ☐No Persistent fatigue ☐Yes ☐No

Cough ☐ Yes ☐ No Spleenomegaly ☐ Yes ☐No

Hepatomegaly ☐ Yes ☐ No Alcohol-induced pain ☐ Yes ☐ No

F. Duration of illness

☐< 1year ☐ 2 years ☐3 years ☐ 4 years

G. Performance status

☐Bedridden; completely dependant

☐Able to walk and clean him/her self with assistance

☐Able to work

H. Case classification

☐ Stage I ☐ Stage II ☐ Stage III ☐ Stage VI

I. Tumor size

☐ 1-2 cm ☐3-5 cm ☐ 6-8 cm ☐ 8-10 cm ☐>10 cm

J. Extent of disease

Laterality ☐ Left ☐ Right ☐Both

Diaphragm ☐ Above ☐Below ☐ Both

K. Date of diagnosis _____

2.2. Any imaging results

X-ray ☐ Yes ☐ No

CT ☐ Yes ☐ No

US ☐ Yes ☐ No

2.3. Type of case

☐ Newly diagnosed ☐ Relapsed ☐ Under treatment

☐ Complete remission ☐ Partial remission ☐ No resposnse

2.4. Treatment

A. Type of treatment:

☐ Radiotherapy ☐ Chemotherapy ☐ Radiotherapy & chemotherapy

B. Number of cycles: ☐ 4cycles ☐ 6cycles ☐ 8cycles

C. Interval between cycles _____

D. treatment response:

☐ Complete response ☐ Partial response ☐ No response

2.5. HIV status ☐ Positive ☐ Negative

3. Risk factors

3.1. Monthly income of the participant or the family of the participant in Ethiopian birr

☐ Low ☐ Middle ☐ High

3.2. Family history with lymphoma

☐ Yes ☐ No

3.3. Is there any history of chronic disease?

☐ Yes ☐ No

3.4. If Q 3.3 is yes, which disease?

☐ If cancer (type)_____

☐ If infection (type)_____

☐ Autoimmune disease (type)_____

☐ Organ transplant

3.5. Is there any co-morbid chronic disease now?

☐ Yes ☐ No

3.6. If Q3.5 is yes, which type of chronic disease?

☐ If cancer (type)_____

☐ If infection (type)_____

☐ Autoimmune disease (type)_____

☐ Organ transplant_____

3.7. Weight_____ Kg

3.8. Do you take any type of tobacco or do you have a history of up taking tobacco?

☐ Yes ☐ No

3.9. If yes, which type of tobacco?

4. Laboratory values

4.1. Complete blood cell count result (CBC)

Neutrophil	☐ Normal	☐ High	☐ Low
Lymphocyte	☐ Normal	☐ High	☐ Low
Eosinophil	☐ Normal	☐ High	☐ Low
Monocyte	☐ Normal	☐ High	☐ Low
Platelets	☐ Normal	☐ High	☐ Low
RBC	☐ Normal	☐ High	☐ Low

4.2. Lactate dehydrogenase (LDH) ☐ Normal ☐ High ☐ Low

4.3. ESR ☐ Normal ☐ High ☐ Low

4.4. Histopathology results

A. Classical: ☐ Nodular Sclerosis ☐ Mixed cellularity

☐ Lymphocyte depleted ☐ Lymphocyte rich

B. Nodular lymphocyte predominant ☐

5. post-treatment reassessment

5.1. Imaging studies

X-ray ☐ Yes ☐ No

CT ☐ Yes ☐ No

US ☐ Yes ☐ No

5.2. CBC count

Neutrophil	☐ Normal	☐ High	☐ Low
Lymphocyte	☐ Normal	☐ High	☐ Low
Eosinophil	☐ Normal	☐ High	☐ Low
Monocyte	☐ Normal	☐ High	☐ Low
Platelets	☐ Normal	☐ High	☐ Low
RBC	☐ Normal	☐ High	☐ Low

5.3. LDH ☐ Normal ☐ High ☐ Low

5.4. Patient status ☐ Complete remission ☐ Partial remission ☐ Relapse