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Epstein-Barr virus and development of PTLD in hematopoietic stem cell transplant recipients: Viral activity and host susceptibility.

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EPSTEIN-BARR VIRUS AND DEVELOPMENT OF PTLD IN HEMATOPOIETIC STEM CELL TRANSPLANT RECIPIENTS: VIRAL ACTIVITY AND HOST SUSCEPTIBILITY

Tese de Candidatura ao grau de Doutor em Patologia e Genética Molecular, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Categoria – Técnico Superior de Saúde

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To my Parents,

Sisters and Brother,

Marco, Diogo and Pedro.

PREFACE

The present study was developed at the Molecular Oncology and Viral Pathology Group, as well at the Virology Service, of the Portuguese Institute of Oncology of Porto FG, E.P.E.

This PhD thesis was written in the form of journal articles. Therefore, we present three articles, with one published and one accepted for publication, included in the attachments, and the third submitted for publication.

The results regarding the retrospective part of this study resulted in the following article: Marinho Dias, J., Lobo, J., Henrique, R., Baldaque, I., Pinho-Vaz., Regadas, L., Branca, R., Campilho, F., Campos Jr, A., Medeiros, R., Sousa, H. Post-transplant lymphoproliferative dirsorder in hematopoietic stem cell transplant patients: A single center retrospective study between 2005 and 2012. *Molecular Medicine Reports*: 2018 Nov;18:4650-4656.

The results regarding the prospective part of this study resulted in the following article, accepted for publication: Marinho Dias, J., Baldaque, I., Pinho-Vaz., Leite, L., Branca, R., Campilho, F., Campos Jr, A., Medeiros, R., Sousa, H. Epstein-Barr virus infection association with allogeneic hematopoietic stem cell transplanted patients in Portugal. *Molecular Medicine Reports (in press)*.

The results from the analysis of susceptibility are presented in the following article, submitted for publication: Marinho-Dias, J., Baldaque I., Pinho-Vaz, C., Regadas, L., Leite, L., Branca, R., Campilho, F., Campos Jr, A., Medeiros, R. and Sousa, H. Single nucleotide polymorphisms as genetic susceptibility markers for Epstein-Barr virus infection and post-transplant lymphoproliferative disorder in hematopoietic stem cell recipients.

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I would like to acknowledge the importance of the Bone Marrow Transplant Service in the development of this project, specially to Dr. Pinho Vaz for its availability and valuable insight, Rute Silva for providing us all the information we needed, and finally to Dr. António Campos Jr., director of Service, for allowing me to do this study.

I have to thank the Anatomical Pathology Service, mainly to Prof. Dr. Rui Henrique for its availability and help provided in the acquisition of biopsy samples, as well as Dr. João Lobo, for its help in sample searching and classification.

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Fortunately, I have a good “lab family” and Sandra and Margarida, provided me a good environment, made me laugh even when I did not want to and assured me that I was skilled to pursue this until the end.

To my aunt Maria Luísa, who ensured that my thesis had to be done even with all the life changes I made throughout these years. I hope I made you proud!

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To my dear husband, Marco, for making me believe in myself, for making me laugh, for all the comfort and quietness, for meeting every need I had throughout these years. Mostly, for giving me the best gift anyone could give, our little snowflake Diogo, I love you dearly. Your support was crucial.

RESUMO

Introdução: Pacientes que recebem transplantes de células progenitoras hematopoiéticas, para tratamento de malignidades, possuem um elevado risco de desenvolver doença linfoproliferativa pós-transplante associada à infeção por EBV. Vários fatores de risco têm sido descritos e a infeção por EBV é uma das maiores complicações após o transplante.

Métodos: O objetivo deste estudo é caraterizar infeções após transplante alogénico de células progenitoras hematopoiéticas e identificar fatores de risco para o desenvolvimento de doenças linfoproliferativas, tendo em consideração caraterísticas clínicas e a suscetibilidade genética do hospedeiro. Desenvolvemos: 1) estudo retrospetivo com 15 pacientes com doença linfoproliferativa pós-transplante diagnosticados entre 2005 e 2012, para caraterização dos fatores de risco de desenvolvimento de doença linfoproliferativa; 2) estudo prospetivo de acompanhamento de 40 pacientes, selecionados aleatoriamente, submetidos a transplante alogénico de células progenitoras hematopoiéticas entre Janeiro e Dezembro de 2015, para avaliação da infeção por EBV em seis períodos diferentes após transplante; e 3) estudo genotípico de polimorfismos em genes associados com a resposta imunológica.

Resultados: O nosso estudo retrospetivo revelou que as doenças linfoproliferativas foram observadas em pacientes que receberam células de dadores não relacionados e que não estavam associadas com o regime de condicionamento. Nestes pacientes, o tempo médio para deteção da infeção por EBV foi de 68 dias e a carga viral média foi de 4.9 $\log_{10}$ cópias/mL. O estudo prospetivo revelou que a infeção por EBV é frequente (70.0%) e ocorre em média 65.6 ± 39.6 dias; A análise revelou que a infeção por EBV foi mais frequente em pacientes com dadores não relacionados (D+60 e D+150 pós-transplante), regime mieloablativo (D+60), ATG (D+150) e GVHD (D+90). A análise de sobrevivência cumulativa demonstrou que pacientes com dadores não relacionado e infeção por EBV levam a taxas de sobrevivência mais baixas (HR=8.8, *p*=0.03). Mortes associadas com transplante compreendem 46.7% dos pacientes e 40.0%, no estudo retrospetivo e prospetivo, respetivamente. No estudo de análise de suscetibilidade genética do hospedeiro, a análise de genótipo confirmou uma associação entre a infeção por EBV e portadores do alelo A de Interleucina-18 rs1143627 (*p*=0.024, OR=14.0); enquanto que o desenvolvimento de doença linfoproliferativa foi associado com o genótipo AA de Interleucina-1 (*p*=0.049, OR=1.28), genótipo GG de Interleucina-10 rs302446 e portadors alelo A de *Mitochondrial Antiviral Signaling* (MAVS) rs6052130 (*p*=0.009, OR=1.50).

Conclusão: A monitorização de EBV é essencial no seguimento do transplante alogénico e o nosso estudo demonstrou que a monitorização deve ser realizada frequentemente e

realizada até ao dia 150 pós-transplante. O estudo também demonstrou que polimorfismos genéticos podem ser úteis como biomarcadores na previsão da infeção por EBV e desenvolvimento de doenças linfoproliferativas pós-transplante nestes pacientes no futuro.

ABSTRACT

Background: Patients receiving hematopoietic stem cell transplants (HSCT), for treatment of hematological malignancies show a considerable high risk of developing EBV-associated posttransplant lymphoproliferative disorder (PTLD). Several risk factors have been described and EBV infection is one of the major complications post-transplantation.

Methods: The aim of this study is to characterize post-allo-HSCT EBV infections and identify risk factors for the development of PTLD, by considering clinical characteristics and host genetic susceptibility. We have developed: 1) retrospective study with 15 patients with PTLD diagnosed between 2005 and 2012 for the characterization of risk factors for PTLD development; 2) a prospective follow-up study with 40 randomly selected patients who underwent allo-HSCT between January and December 2015 that were evaluated for EBV infection at 6 different times after transplant; and 3) a genotyping study of polymorphisms in genes associated with host immune response.

Results: Our retrospective study reveals that PTLD was observed in patients receiving cells from unrelated donors but was not associated with the type of conditioning regimen. In these patients, the mean time for the EBV infection detection was of 68 days and the mean viral load of 4.9 $\log_{10}$ copies/mL. The prospective study revealed that EBV infection is frequent (70.0%) and occurs in mean time of 65.6 ± 39.6 days; the analysis revealed that EBV infection was more frequently found in patients with unrelated donors (D+60 and D+150 post-transplant), myeloablation (D+60), ATG (D+150), GVHD (D+90). Analysis of cumulative survival showed that unrelated donor and EBV infection lead to poor survival rates (HR=8.8, p=0.03). Transplant related rates of death are from 46.7% and 40.0%, in the retrospective and prospective study, respectively. In the study regarding host genetic susceptibility, the genotype analysis confirmed an association between EBV infection and Interleukine-18 (IL-18) rs1143627 Acarriers (p=0.024, OR=14.0); while PTLD development was associated with Interleukine-1 (IL-1A) rs2856838 AA genotype (p=0.049, OR=1.28), Interleukine-10 (IL-10) rs302446 GG genotype (p=0.037, OR=7.20) and Mitochondrial Antiviral Signaling (MAVS) rs6052130 Acarriers (p=0.009, OR=1.50).

Conclusion: EBV monitoring is essential in the subset of allogeneic transplant and our study demonstrated that monitoring should performed frequently and extended until day 150. The study also shows that host genetic polymorphisms could be useful as biomarkers for the prediction of EBV infection and PTLD development in these patients in the future.

ABREVIATIONS

AA – Aplastic Anemia

AdV – Adenovirus

aGVHD – Acute Graft Versus Host Disease

aHSCT – Allogeneic Hematopoietic Stem Cell Transplant

ALL – Acute Lymphoid Leukemia

AML – Acute Myelogenous Leukemia

ATG – Anti-Thymocyte Globulin

ATL – Adult T-cell Lymphoma

BCL – B-Cell Lymphoma

BKV – BK Virus

BL – Burkitt Lymphoma

BM – Bone Marrow

Bu – Busulfan

cGVHD – Chronic Graft Versus Host Disease

CLL – Chronic Lymphoid Leukemia

CML – Chronic Myelogenous Leukemia

CMV – Cytomegalovirus

CNS – Central Nervous System

CRP – C-Reactive Protein

CTL – Cytotoxic T-Cell

Cy – Cyclophosphamide

DLBCL – Diffuse Large B-Cell Lymphoma

DNA – Deoxyribonucleic acid

EA – Early Antigen

EBER – Epstein-Barr virus-encoded small RNA

EBNA – Epstein-Barr virus Nuclear Antigen

EBV – Epstein-Barr Virus

EDTA - Ethylenediamine Tetra-acetic Acid

FFPE – Formalin-Fixed Paraffin-Embedded

Flu – Fludarabine

GVHD – Graft Versus Host Disease

HBV – Hepatitis B Virus

HHV – Human Herpesvirus

HIV – Human Immunodeficiency Virus

HL – Hodgkin Lymphoma

HLA – Human Leukocyte Antigen

HPV – Human Papilloma Virus

HRS – Hodgkin Reed-Steinberg-like cells

HSC – Hematopoietic Stem Cells

HSV – Herpes Simplex Virus

HTLV – Human T-cell Leukemia Virus

IFN – Interferon

IFNG – Interferon Gamma

IFNGR – Interferon Gamma Receptor

Ig - Immunoglobulin

IL - Interleukin

IM - Infectious Mononucleosis

IR – Immune Reconstitution

ISH – *In situ* Hybridization

LD – Lineage Disequilibrium

LDH – Lactate Dehydrogenase

LMP – Latent Membrane Protein

LP – Leader Protein

MA – Myeloablative

MAVS – Mitochondrial Antiviral Signaling

MDS – Myelodysplastic Syndrome

Mel - Melphalan

MM – Multiple Myeloma

NCI – National Cancer Institute

NHL – Non-Hodgkin Lymphoma

NIH – National Institute of Health

NK – Natural Killer

NMA – Non-Myeloablative

PBMC – Peripheral Blood Mononuclear Cell

PBSC – Peripheral Blood Stem Cell

PCR – Polymerase Chain Reaction

PTLD – Post-Transplant Lymphoproliferative Disorder

REAL – Revision European-American Lymphoma classification

RFLP – Restriction Fragment Length Polymorphism

RI – Reduction of Immunosuppression

RIC – Reduce Intensity Conditioning

RNA – Ribonucleic Acid

RT-PCR – Real-Time Polymerase Chain Reaction

SEER – Surveillance, Epidemiology and End Results Program

SNP – Single Nucleotide Polymorphism

SOT – Solid Organ Transplant

TBI – Total Body Irradiation

TGF – Tumor Growth Factor

TNF – Tumor Necrosis Factor

UCB – Umbilical Cord Blood

VZV – Varicella Zoster Virus

WHO – World Health Organization

FIGURES

Study I

Study II

TABLES

Study I

Study II

Study III

INDEX

INTRODUCTION

1. Cancer

1.1 Epidemiology

Cancer is the second most common cause of death, following heart disease, and is an emerging public health issue in developed countries. According to the World Health Organization (Spolverato, Kim et al.) cancer figures amongst the leading causes of morbidity and mortality with approximately 14 million new cases and 8.2 million deaths in 2012 (Figure 1) (data from WHO fact sheet 2014). In Portugal, in the same year, an overall of 49174 new cases and 24112 deaths were reported.

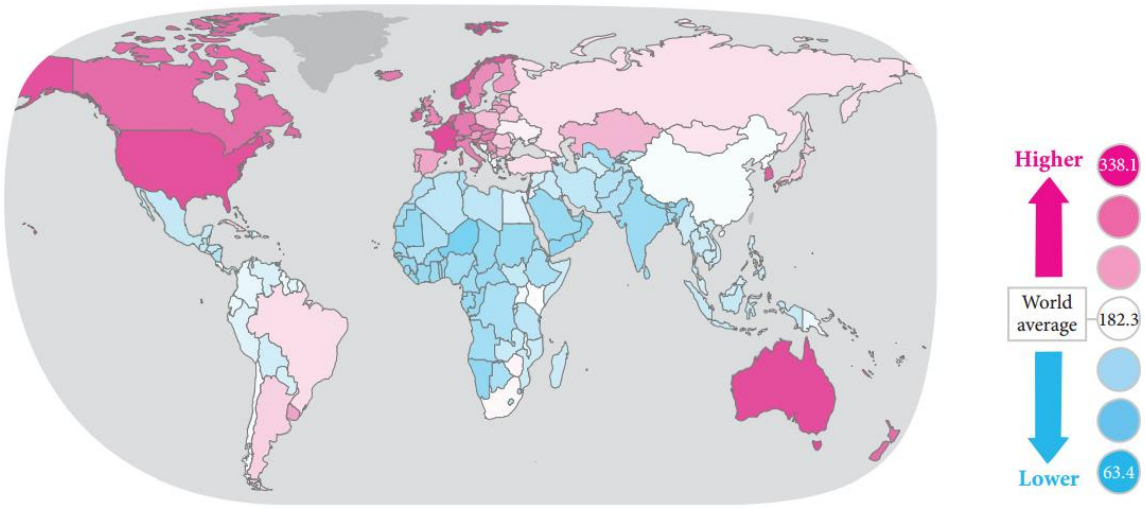

Figure 1: Age standardized incidence rates per 100,000 population compared to the world average (IARC 2014).

Despite its incidence, mortality rates have been decreasing with the introduction of new screening methods, anti-cancer drugs and better treatment options. Currently, there are over 30 million cancer survivors worldwide and rates of survival are continuing to increase, including in Hodgkin's disease or children's leukemia with survival rates reaching 90% (Moser and Meunier 2014).

Considering that cancer occurs in approximately one of three individuals and that DNA mutations arise at a frequency of one in 20 million per gene per cell division, it would be expected that human populations anywhere in the world should show parallel incidence frequencies (Hejmadi 2010). However, cancer incidence rates vary across countries, which indicates the influence of different factors in cancer incidences of different populations. The major risk factors for cancer development include tobacco, alcohol consumption, unhealthy

diet, obesity and sedentarism (Singh and Dorak 2017). In addition, chronic infections have been described as having an increasing role in cancer development, with approximately 15% of all cancers to be associated with *Helicobacter pylori*, *Human Papilloma Virus* (HPV), Hepatitis B and C, as well as Epstein-Barr Virus (EBV) (Khan, Afaq et al. 2010, Plummer, de Martel et al. 2016). Furthermore, some populations carry cancer-susceptibility genes or that the environment where they live contributes to the cancer incidence rates (Wilson, Jones et al. 2002, Hejmadi 2010).

1.2 Carcinogenesis

Cancer is a disease in which a group of abnormal cells grow uncontrollably. In normal cells, the cell cycle regulation controls the proliferation of cell and is dependent on signals that either trigger or inhibit cell division, differentiation or death (Hejmadi 2010, Peterson and Kovyrshina 2017). Cancer cells are able to be partially independent of this regulation, and therefore cells are maintained in a continuous growth which allows the invasion of surrounding tissues, dissemination of the tumor cells (metastasis) and ultimately to death (Wilson, Jones et al. 2002, Hejmadi 2010, Pandya, Orgaz et al. 2017).

The initiation and progression of cancer is dependent on external (tobacco, chemicals, radiation and infectious microorganisms) and internal factors (inherited mutations, hormones, immune conditions and mutations associated with metabolism) which combined result in the unrestrained cell proliferation. Multiple genetic changes are necessary for the development of most cancers, and indeed the majority of cancers take months to years to result in a detectable cancer (Alberts, Johnson et al. 2002). These data are supported by the evidence that cancer incidences are well correlated with the exponential increase of age (Malaguarnera, Cristaldi et al. 2010). Cancer is a genetic disease associated to the accumulation of genetic modifications in cells, therefore the longer the lifetime, the higher the risk of developing cancer (Hejmadi 2010, Zou, Wang et al. 2017).

2. Lymphoma and Leukemia

2.1 Incidence

According to the *National Cancer Institute* (NIH) there are estimated over 60 thousand new Leukemia cases in 2017 and approximately 24,500 deaths worldwide (Figure 2). Currently, leukemia occupies the 9th position of most common types of cancers and is most frequently diagnosed in people aged from 65 to 74 years-old (22.4%) (Howlader, Noone et al. 2017).

The distribution of leukemias by types varies with age: children are generally affected by acute lymphocytic leukemia (ALL), which is also observed in adolescents and young adults; and the elderly populations are more often affected by chronic lymphoid leukemias (Bhayat, Das-Gupta et al. 2009). Despite its severity, ALL is mostly curable and 5-year survival in children has reached over 90% in optimum conditions, with improvements in adolescents and adults. This has been improved with the use of allogenic hematopoietic stem cell transplants (aHSCT) and immunotherapy, as well as using pediatric inspired regimens in young adults (Pui, Pei et al. 2011, Ibrahim, Ali et al. 2014, Malvezzi, Carioli et al. 2016).

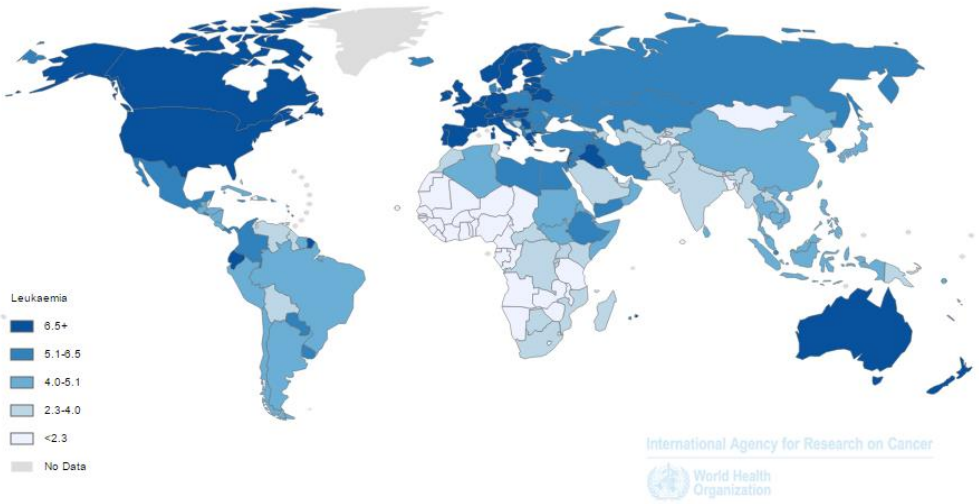

Figure 2: Worldwide incidence of leukemia (Ferlay, Soerjomataram et al. 2013).

Lymphomas are often divided in Hodgkin (HL) and non-Hodgkin lymphoma (NHL). HL has an estimated 8,260 new cases in 2017 with approximately one thousand deaths (Figure 3) and the 5-year relative survival is estimated in 86.4%, depending on stage of the disease. HL is more common in young adults, and more frequent in men. The median age of diagnosis is 39 years-old, with 31.3% of these cases being diagnosed between 20 and 34 years-old. Non-Hodgkin lymphoma is more frequent than HL, with 72,240 new cases expected for 2017 and approximately 20,000 deaths (Figure 4). The 5-year survival rates can reach up to 71% depending on stage at time of diagnosis, mainly due to the fact that 50% of cases are diagnosed with disease at distance. Median age at diagnosis is 67 years-

old, with diagnosis being more frequent between 65 and 74 years-old (Howlader, Noone et al. 2017).

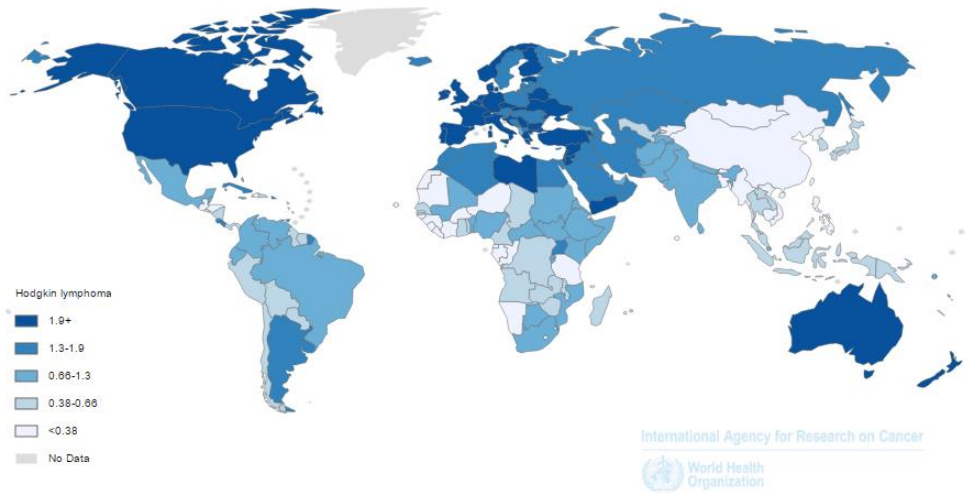

Figure 3: Worldwide incidence of Hodgkin lymphoma (Ferlay, Soerjomataram et al. 2013).

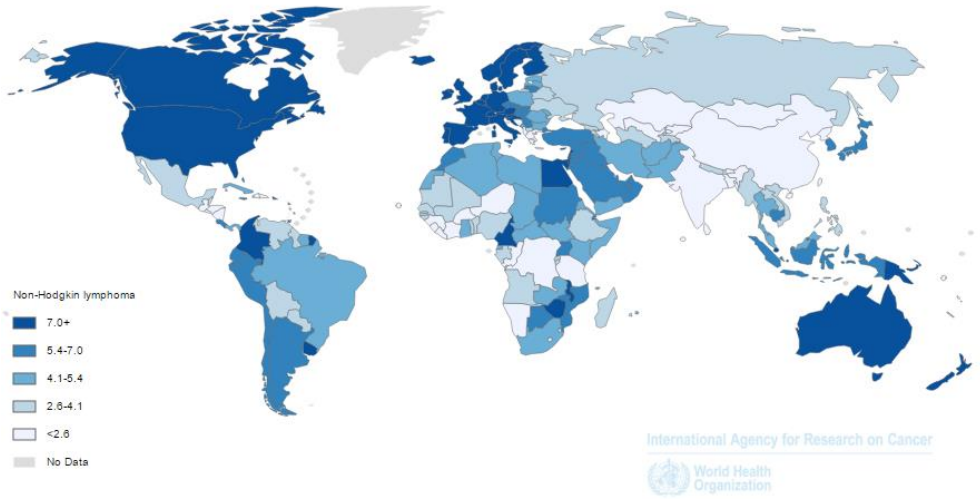

Figure 4: Worldwide incidence of non-Hodgkin lymphoma (Ferlay, Soerjomataram et al. 2013).

2.2 Pathology

The bone marrow is the main source of hematolymphoid cell populations (Baum, Weissman et al. 1992). The bone marrow is filled with stem cells that can self-renew and differentiate into different lineage precursors, which are defined as hematopoietic stem cells (Yoon, Bhsc et al.). The hematopoiesis is a process dependent upon division and differentiation of HSC, that can give rise to all blood lineages of the adult organism (Figure 5) (Passegue, Jamieson et al. 2003).

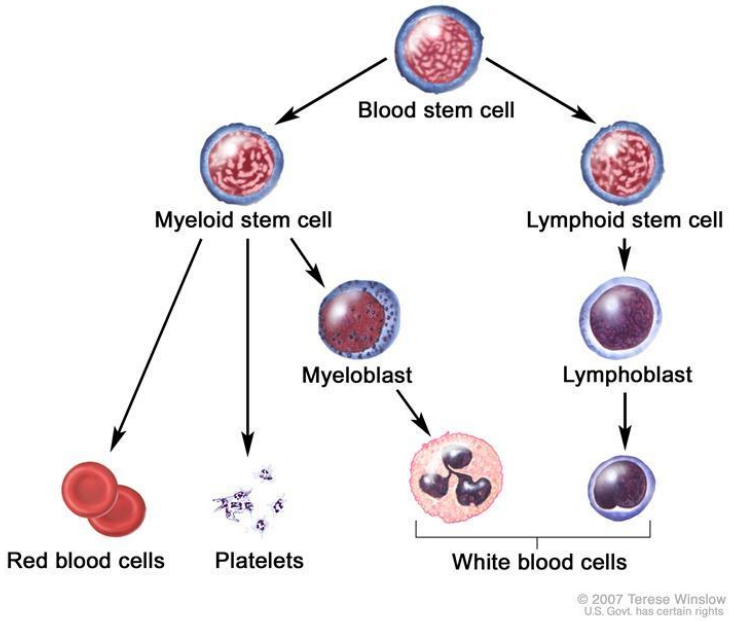

Figure 5: Blood cell development (National Institute Health, IARC).

HSC are commonly divided in two sub-groups: 1) long-term cells that can divide themselves indefinitely; and 2) short-term cells who have a limited interval of self-renewal. Recent studies verified that both stem cells and cancerous cells have the ability to self-renew and that several pathways, associated with cancer development, are also associated with stem cell regulation/development (Passegue, Jamieson et al. 2003).

The hematopoietic and lymphatic tissues are crucial in the maintenance of vital functions of host defense (Janeway, Travers et al. 2001). All cells from these tissues are descendant from the same stem cell population and in their functional activities interact in multiple ways. Because of this connection between them, disorders in one cell population may consequently cause reactions to others (Buja and Krueger 2013, F. Krueger 2013). The presence of detailed genetic abnormalities, or the demonstration of tumor genetic homogeneity of a cell population can lead to a clonal pathologic process. Moreover, molecular methods are used to sub-classify these neoplasms (McPherson and Pincus 2016). Specific genetic abnormalities in hematolymphoid malignancies have significant

prognostic value, which in turn can influence the type of treatment and the clinical outcome (Jeon, Yoon et al. 2017). Malignant neoplasia of the lymphatic tissues are often acknowledged as malignant lymphomas, or when malignant cells circulate through blood, lymphocytic leukemias (Janeway, Travers et al. 2001, McPherson and Pincus 2016).

2.3 Subtypes of Lymphomas

There are two groups of malignant lymphomas: Hodgkin's lymphomas (HL), also known as lymphogranulomatosis; and non-Hodgkin lymphomas (NHL). HL is different from NHL by its polymorphic features, including certain inflammatory components such as fibrosis and occasional regression (Buja and Krueger 2013) and although it is a lymphatic malignancy, it is accompanied by a large number of associated non-neoplastic cells, which may influence the course and progression of the disease (PDQ1 2017). NHL, by contrast, begins as malignant clonal proliferations. Conversion from HL to NHL and combinations of HL with certain types of NHL have also been observed (Buja and Krueger 2013, PDQ2 2017).

The diagnosis of a malignant lymphoma as either HL or NHL and its classification based on histologic, immunologic and cytogenetic markers, contributes to the correct staging of the disease and determine its treatment and the life expectancy (Jaffe, Harris et al. 2008). Staging of all lymphomas is similar: *stage I* indicates involvement by lymphoma of 1 lymph node site (axillary, neck); *stage II* indicates involvement of 2 lymph node sites on the same side of the diaphragm (neck and axillary, or left and right inguinal); *stage III* indicates involvement of lymph nodes on both sides of the diaphragm; and *stage IV* indicates involvement of lymphatic and extralymphatic sites such as liver, spleen, bone marrow. Malignant lymphomas may also arise from sites other than the lymph nodes and these are grouped together as extranodal lymphomas and have different staging (Buja and Krueger 2013, Cheson 2015).

Hodgkin lymphoma is characterized histologically by mixed proliferations of lymphoid cells with various numbers of histiocytes, eosinophils, and the diagnostic Hodgkin cells or Reed-Sternberg cells (HRS) (Pileri, Ascani et al. 2002). Lymph nodes may show focal or diffuse involvement with altered architecture and invasion beyond their capsule. HL cells are mononuclear histiocytoid blasts with vesicular nuclei and large prominent nucleoli. Reed-Sternberg cells are essentially similar but binucleated blasts (Ansell 2016). The classification system of HL depends on the presence/absence of HRS, or lymphocytic and histiocytic cells (Kennedy-Nasser, Hanley et al. 2011). Therefore, classification of HL is divided into 4 major groups according to their overall cell composition: lymphocyte-predominant type, mixed-cellularity type, nodular-sclerosing type, and lymphocyte-depleted

type. The most frequently affected lymph nodes are in the mediastinum (59%), the neck (55-58%), the axillae (13-14%), and the lung hilus (11-12%) (Buja and Krueger 2013).

Non-Hodgkin lymphomas are a diverse group of B, T and natural killer (NK) cell lymphomas (Ansell 2015). These lymphomas arise from mature and precursor cells and typically, infiltration of both lymphoid and hematopoietic tissues is observed, but extension to other organs is also possible. The etiology of most NHLs is unknown, nevertheless some have been associated with viral infections: Epstein-Barr Virus (EBV) and Burkitt's and Burkitt's-type lymphoma; and Human Herpesvirus 8 (HHV-8), Human T-cell Leukemia Virus Type 1 (HTLV-1) and adult T-cell leukemia (ATL) (Buja and Krueger 2013, Linch and McNamara 2016, PDQ2 2017).

2.4 Subtypes of Leukemias

Leukemia is a common malignancy, affecting all age groups, that begins in blood-forming tissues (Davis, Viera et al. 2014). Leukemias are divided into four major sub-groups according to the French-American-British classification (Lilleyman, Hann et al. 1986, Abdul-Hamid 2011): acute lymphoid leukemia (ALL), acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML) and chronic lymphoid leukemia (CLL). Chronic leukemia has a slow progression and abnormal cells are not able to function, while acute leukemia has a more aggressive form and cells are functional at the stage they are arrested (Torkaman, Charkari et al. 2011).

Suspicion of leukemia should arise when white cell count is elevated, signs of bleeding tendency (petechia, purpura or bruising), lymphadenopathy, hepatosplenomegaly, expiratory wheeze (due to mediastinal mass), anemia, thrombocytopenia and thrombocytosis. Infection, inflammation and stress must also be considered, since these factors alter blood count (Raab and Gartner 2009, Davis, Viera et al. 2014).

Chronic myelogenous leukemia (CML) is associated with a characteristic chromosomal t(9,22)(q34;q11) translocation, also known as the Philadelphia chromosome and is defined by myeloid hyperplasia, leukocytosis, basophilia, and splenomegaly. Clinical characteristics are fatigue, weight loss, sweats, bone pain, anemia, hepatosplenomegaly, and petechial hemorrhages (Jabbour and Kantarjian 2016). The life expectancy of patients with CML depends on disease progression and type of treatment; 45% to 65% of patients survive 5 years (Buja and Krueger 2013, Jabbour and Kantarjian 2014).

Acute Myelogenous Leukemia (AML) is a myeloproliferative disease representing approximately 90% of all acute leukemias and is frequently observed in patients older than

65 years-old (NCI 2012). Patients typically present with malaise and fatigue, may have resistant skin infections, unusual pallor, and bleeding from the gums and the nose; blood smears show leukopenia, or excessive leukocytosis, with increase in immature cells; liver and the spleen are enlarged and infiltrated by atypical blasts; and additional symptoms result from metabolic and electrolyte derangements (hypokalemia, hypercalcemia), agranulocytosis (necrotizing enterocolitis), or rapid lysis of leukemic blasts (Buja and Krueger 2013). Survival rates for all AML subtypes combined are 40% at 15 months and approximately 20% at 50 months (Buja and Krueger 2013). AML can be classified in eight subtypes, according to the French-American-British (FAB) classification: AML-0 to AML-8 (Figure 6) (Rasaiyaah, Yong et al. 2007, Plesa, Ciuperca et al. 2008, NCI 2012).

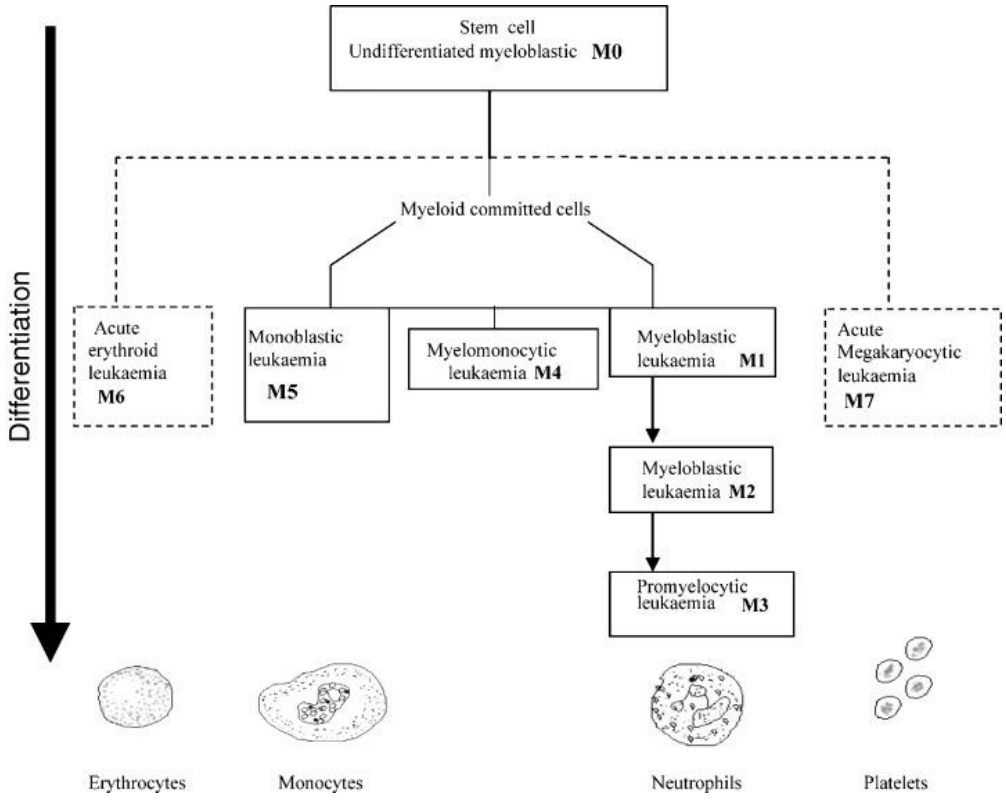

Figure 6: Schematic diagram of the myeloid lineage illustrating the different types of acute myeloid leukemia (Rasaiyaah, Yong et al. 2007).

Lymphocytic leukemias are characterized by blood circulating malignant cells and lymphomatous infiltration of bone marrow, lymphatic organs, or extralymphatic sites. Chronic Lymphocytic Leukemia (CLL) is observed in approximately 90% of B-cell malignancies. This disease has a slow progression and predisposes patients to infections due to immunodeficiency and autoimmune reactions (Hallek 2013). Acute lymphocytic

leukemia (ALL; cytological subtypes L1-L3 according to cell size) is frequently a childhood leukemia, with over 80% of cases consisting of monoclonal B-precursor cells, and approximately 15% of cells from the T-cell lineage (Cooper and Brown 2015). Clinical characteristics are anemia (pallor, fatigue), thrombocytopenia (hemorrhage), and mature leukocytopenia. The combination of chemotherapy and radiation therapy led into a long-term disease-free survival of 70% to 80% of children (Buja and Krueger 2013, Chiaretti, Zini et al. 2014).

Aplastic anemia (AA) is an anemia of deficient hematopoiesis and is characterized by anemia, neutropenia, and thrombocytopenia, and may progress to leukemia (Scheinberg 2012). Patients often appear pale, with petechial hemorrhages, and increased susceptibility to infection. Resistant cases necessitate multiple transfusions and thus may be complicated by iron overload syndrome with secondary hemochromatosis, cardiac failure, and diabetes mellitus (Scheinberg and Young 2012, Buja and Krueger 2013, Savona, Malcovati et al. 2015).

Myelodysplastic syndrome is a type of hematopoietic hyperplasia and dysplasia with peripheral cytopenia. MDS originates from hematopoietic stem cell defects with multiple genetic abnormalities and clonal proliferation of hematopoietic cells, T lymphocytes, and clonal or polyclonal B lymphocytes. Several stages are identified by analyzing blasts population and maturation (Germing, Kobbe et al. 2013). Anemia and fatigue are early symptoms, followed by neutropenia, infections, thrombocytopenia, and bleeding. Bone marrow aspirates show a megaloblastic erythropoiesis with ring sideroblasts, increased myeloblasts, and hypolobulated megakaryocytes. Transition to acute myelogenous leukemia (AML) occurs in 40% to 50% of advanced cases (Buja and Krueger 2013, Garcia-Manero 2015).

Multiple myeloma (MM) also recognized as plasmacytoma is a neoplastic clonal proliferation of plasmocytic cells usually at multiple sites in the bone marrow. It is frequently accompanied by the production of unusual immunoglobulin components (gammopathy, monoclonal M protein in serum, and Bence Jones protein in urine). About 90% of patients will develop osteolytic bone lesions, due to high osteoclastic and low osteoblastic activity, which is a major cause of morbidity and mortality of these patients (Miceli, Colson et al. 2011). Clinical features include bone pain, anemia, bleeding, hypercalcemia, hyperglobulinemia, and susceptibility to infection. Anemia occurs in approximately 75% of these patients and is the major cause for fatigue (Buja and Krueger 2013, Rajkumar and Kumar 2016).

3. Hematopoietic Stem Cell Transplantation

3.1 Transplantation

Transplant of hematopoietic stem cells is currently the standard treatment for patients with congenital or acquired hemoglobinopathies or with malignancies sensitive to chemotherapy, radiotherapy or immunological treatments (Gratwohl, Baldomero et al. 2010). Allogeneic stem cells are infused to correct the basic genetic defect by replacing genes required for a normal hematopoiesis. For a successful treatment, two main objectives are required: 1) elimination of the deficient marrow; and 2) providing a tolerant environment for the transplanted marrow survival (Lucarelli, Isgro et al. 2012).

Conditioning for immunological system suppression is required and therefore there are two types of approaches: myeloablative conditioning (MA) and non-myeloablative/reduced-intensity conditioning (RIC). MA involves induction of long-lasting aplasia and is achieved with total body irradiation and/or alkylating agents. Some examples of MA conditioning are the use of cyclophosphamide (CY)/total body irradiation (TBI) or busulfan (BU)/CY; NMA conditioning consists in applying fludarabine (FLU)/TBI or low dose of TBI; finally, RI is often applied using FLU and melphalan (MEL), FLU/BU and FLU/CY. Variations in these regimens may occur depending on which center they are applied (Juric, Ghimire et al. 2016).

The success of HSCT is often associated with the HLA-match of hematopoietic stem cells. Indeed, HLA-related sibling would be the ideal donor with 25% chance to be HLA-identical (Petersdorf 2007). Patients without a related match must search for unrelated donors, always considering variations in HLA-specific alleles and ethnicity. Nevertheless, for patients lacking any HLA-match unrelated donor, three other options are available: HLA-mismatched unrelated donors, umbilical cord blood (UCB) and HLA-haploidentical family members (Gyurkocza, Rezvani et al. 2010).

The source of stem cells is also an important issue in HSCT, since it depends on the age of the donor and recipient, clinical comorbidities, stage of disease and is extremely variable between centers according to their experience (Juric, Ghimire et al. 2016). Three options are available for harvesting HSC: Bone marrow (BM), Peripheral blood stem cells (PBSC) and UCB: BM gives a lower risk of GVHD occurrence but has a more invasive harvesting process; PBSC does not require general anesthesia for collection and has minimal secondary effects, is related to faster engraftment and immune reconstitution (IR) but carries a higher risk for GVHD; and UCB, is a non-invasive procedure, has lower risks of GVHD and relapse, although a lower number of HSC are available and a slower IR is observed (Smith and Wagner 2009, Juric, Ghimire et al. 2016).

3.2 Post-transplant complications

Post-transplant infections and graft-versus-host disease (GVHD) are the most common problems in HSCT, especially in those patients receiving an allogeneic transplant (aHSCT) (Choi, Levine et al. 2010).

Among the infections that affect patients undergoing aHSCT, viruses are the most frequent, since they require a fast and effective immune response (Figure 7). The list of viruses that infect these patients and cause severe morbidity and mortality gets longer each day, nevertheless Cytomegalovirus (CMV) is still the most important virus (Atalay, Gokahmetoglu et al. 2014).

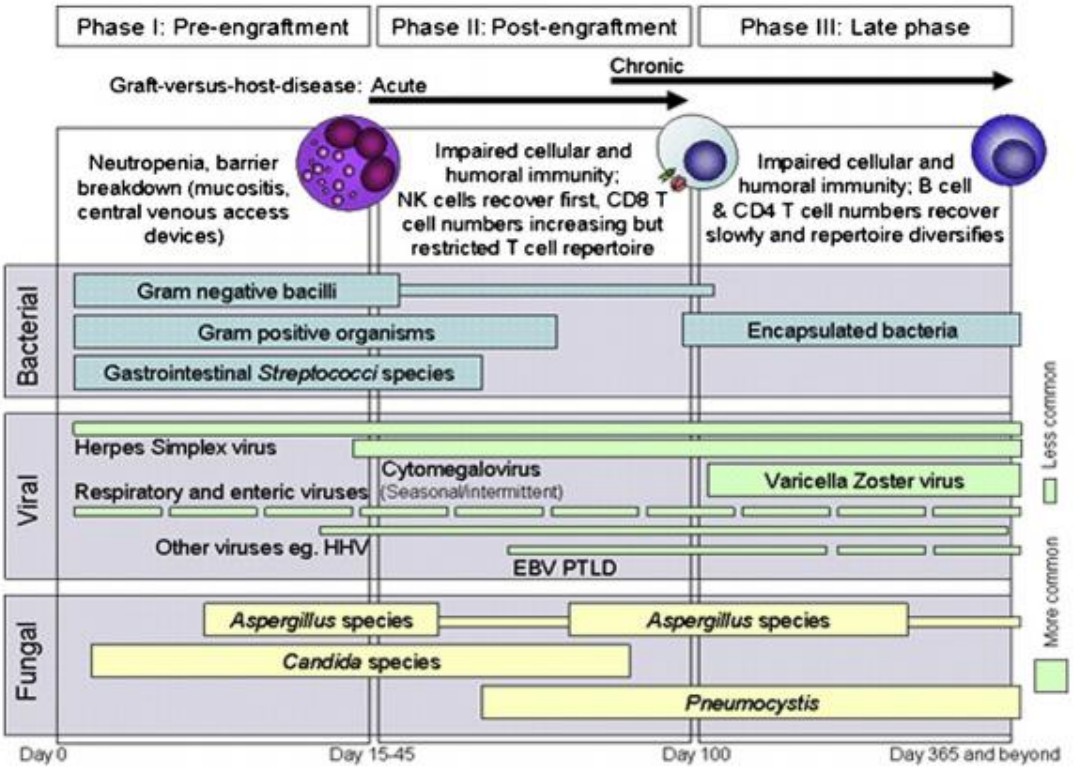

Figure 7: Infections following allogeneic hematopoietic stem cell transplantation (Tomblyn, M. *et al*., 2009).

GVHD is characterized by the rejection of the graft by the host due to an immune rejection of the host tissues led by the donor lymphocytes (Barriga, Ramirez et al. 2012). Several studies have shown that over 50% of patients who undergo aHSCT may develop GVHD (Funke, Moreira et al. 2016). Transfused immunocompetent T lymphocytes recognize and destroy such allogeneic host cells (epidermal, hepatocytes, intestinal, and hemolymphatic tissues). Microscopically, a typical acute GVHD shows a T-cell immune reaction in the skin, the liver, and the upper intestines combined with growth inhibition and atrophy of

hemolymphatic tissues. Severe acute GVHD has a high mortality secondary to severe ulcerating enteritis with superinfection, diarrhea, and fluid loss; severe hepatitis with hepatocellular necrosis; or systemic viral disease and bacterial septicemia (Buja and Krueger 2013, Socie and Ritz 2014).

Another severe complication of HSCT is the development of post-transplant lymphoproliferative disorder (PTLD). PTLD occurs after either solid or HSCT and is responsible for high rates of morbidity and mortality among these patients (Zimmermann and Trappe 2013). In HSCT recipients the incidence of PTLD is 1.0%, in non-complicated HLA-matched transplants, and 25% after T-cell depleted highly immunosuppressed transplants (Capello and Gaidano 2009). PTLDs are characterized by several factors: usually derive from B cells, with preferential presentation as non-Hodgkin's lymphoma; usually originate in extranodal sites; rarely affect skin; aggressive behavior; and frequently are Epstein-Barr virus related (Bar-Natan and Nagler 2006).

In aHSCT a regimen of immunosuppression must be applied to avoid graft rejection. Considering a reduced intensity conditioning (RIC) and myeloablative regimen (MA), several studies suggest that patients subjected to RIC carry more high-risk features and comorbidities, although overall survival rates were found to be similar (Chevallier, Szydlo et al. 2012, Atilla, Atilla et al. 2017). Myeloablative condition comprises the solo/combined utilization of the following compounds: cyclophosphamide, busulfan, anti-thymocyte globulin, fludarabine and/or melphalan. Most of RIC include fludarabine and intermediate doses of busulfan and melphalan. RIC regimens are associated with mild myelosuppression, low-treatment related toxicity and wider antitumor responses (Atilla, Atilla et al. 2017). Almost all patients receiving myeloablative conditioning regimens develop fever during neutropenia, and most of these febrile episodes are due to infections (Satwani, Baldinger et al. 2009, Therriault, Wilson et al. 2010, Safdar 2011). Infections in neutropenia after aHSCT may be life-threatening. Bacterial pathogens account for about 90% of infections during this phase (Figure 7) (Safdar 2011, Balletto and Mikulska 2015).

4. Post-Transplant Lymphoproliferative Disorder

4.1 History

Although post-transplant lymphoproliferative disorder (PTLD) is relatively rare, it is the most frequent malignant disease early after transplantation (Glotz, Chapman et al. 2012). PTLD refers to a heterogeneous group of lymphoproliferative diseases, with potentially life-threatening conditions, exhibiting a spectrum of histopathologies (Gulley and Tang 2010, Mucha, Foroncewicz et al. 2010, Glotz, Chapman et al. 2012). These lymphoproliferative disorders, may be nodal and/or extranodal, restricted to the allograft or widely disseminated (Allen, Alfieri et al. 2002).

PTLD incidence seems to increase in patients receiving either intense immunosuppression to protect against GVHD and/or increased immunosuppression following identification of de novo human leukocyte antigen (HLA) antibodies in long-term transplant recipients. The clinical, morphologic, and biologic heterogeneity of PTLD has made difficult the understanding of its development and the treatment of these complex disorders.

The lymphoid proliferations that occur after organ transplantation have been recognized for more than a quarter of century (Castellano-Sanchez, Li et al. 2004). In 1981 Frizzera et al. studied tumors from a group of renal transplant recipients and observed the occurrence of lymphoproliferations that had not been described before (Frizzera, Hanto et al. 1981). Given the heterogeneity in tumor cell size and shape he called them “polymorphic” and additional investigation demonstrated that tumors were composed of B-lymphocytes. Frizzera et al. then created a classification system which differentiated nonspecific reactive hyperplasia from polymorphic diffuse B cell hyperplasia and polymorphic diffuse B cell lymphoma and from immunoblastic sarcoma (Frizzera, Hanto et al. 1981, Kalinova, Indrakova et al. 2009).

Later, in 1988, Nalesnik et al. investigated a transplant population at the University of Pittsburg and rearranged the old classification system: by not distinguishing the clinical presentation of the two types of polymorphic lesions, both were included under the term polymorphic PTLD; and separating the group of lesions which resemble typical non-Hodgkin’s lymphomas in occurrence and aggressive behavior designated as monomorphic PTLD. In 1995, Knowles *et al*. established a new classification with three categories: 1) reactive hyperplasia of plasma cells; 2) polymorphic hyperplasia and polymorphic lymphoma, both of which were monoclonal and lacked oncogene and tumor suppressor gene alterations; and 3) true lymphomas and hematopoietic neoplasms which were monoclonal and contained proto-oncogenes and/or tumor suppressor gene alterations (Knowles, Cesarman et al. 1995, Kalinova, Indrakova et al. 2009). Then, in 1997 the *Society for Hematopathology Workshop Classification* categorized PTLDs into: early lesions,

polymorphic PTLDs, monomorphic PTLDs (B and T cell lymphomas), plasmacytoma-like lesions, and T cell-rich large B cell lymphoma/Hodgkin's disease-like lesions. In 2001, Harris, Swerdlow, Frizzera and Knowles reviewed the classification system for the 2001 World Health Organization Classifications of Tumors, although, doubts about the extent to which specific genetic or molecular alterations, remained. Therefore, a last update was performed, in 2008, where WHO expanded the definition of disease by considering patients' age at diagnosis, tumor location, molecular characteristics, association with viral infection and inflammation, as a criteria (Kalinova, Indrakova et al. 2009, Turner, Morton et al. 2010, Campo, Swerdlow et al. 2011).

4.2 Incidence

The cumulative incidence of PTLD in allogeneic hematopoietic stem cell transplantation (HSCT) recipients is 1.0% (range 0.5-1.8%), with slightly higher rates in the pediatric population (Castellano-Sanchez, Li et al. 2004, Grywalska, Markowicz et al. 2013). Overall incidence of PTLD varies from 1 to 22% depending on what type of organ was transplanted, patient age, EBV serostatus from recipient and donor, aggressiveness of immunosuppression and combination of risk factors (Bar-Natan and Nagler 2006, Ibrahim and Naresh 2012) .

Rate of survival depends mainly on patient age and extent of disease, with pediatric patients and patients with localized disease having a better prognosis. The more aggressive are monomorphic lesions (Kalinova, Indrakova et al. 2009, Kim, Kim et al. 2010, Luo, Zhang et al. 2014). .

4.3 Development

PTLD is usually classified as early onset lesions, which develop within one year, and late onset lesions, which occur later than 1-year post-transplant. PTLD pathogenesis is multifactorial and EBV plays a major role in the development, by driving the proliferation of infected B cells (Ibrahim and Naresh 2012). Knowing the anatomic distribution of PTLD is important for diagnosis (Tai, Tirumani et al. 2015).

The anatomic distribution of PTLDs varies with patient age and the type of immunosuppressive therapy. PTLD localizes specially in the area of the transplanted organ or in the allograft itself. For HSCT patients, PTLD tends to be disseminated and affects mainly lymph nodes (Tai, Tirumani et al. 2015, Metser and Lo 2016). Childhood PTLDs often involve lymphoid tissues including lymph nodes and adenoids and arise in the

abdomen, thoracic cavity, and head and neck; while PTLDs in adults tend to localize to the liver, lung, lymph nodes, and gastrointestinal tract (Castellano-Sanchez, Li et al. 2004).

Risk factors include young age and age over 50 years at transplantation, white race, unrelated or HLA-mismatched graft, Epstein-Barr virus negative serostatus prior to transplant, primary EBV infection, type of organ transplant, intensity of immunosuppression and presence of cytomegalovirus disease (Kim, Kim et al. 2010, Glotz, Chapman et al. 2012). EBV infection is thought to play the most important role in the pathogenesis of PTLD, although, not all PTLD cases are EBV related (Kim, Kim et al. 2010, Luo, Zhang et al. 2014). To this date, there have been no large studies performed to explore the connection of EBV infection, CMV infection, and acute rejection. It is therefore difficult to define the relative contribution of these events as separate risk factors for PTLD (Glotz, Chapman et al. 2012). An elevated EBV-DNA load has recently become a sensitive aid for predicting individual patients at risk for PTLD development (Luo, Zhang et al. 2014).

Current data for several studies suggests that PTLD is likely to be associated with a high level of immunosuppression rather than the individual use of immunosuppressive agents. Moreover, results from separate studies demonstrate that transplant recipients treated with triple or quadruple combinations of immunosuppressive agents are at higher risk of developing PTLD than patients receiving less agents (Glotz, Chapman et al. 2012). These findings imply that a reduction in immunosuppressive load may lead to a decline in the risk of PTLD (Loren, Porter et al. 2003). The results of some studies suggest that lengthy immunosuppression might also increase the risk of PTLD (Issa, Amer et al. 2009). It has also been suggested that the balance of T- and B-cell depletion may have an impact on the risk of PTLD, although the optimal balance has not been determined (Herzig, Juffs et al. 2003, Opelz and Dohler 2004, Kremers, Devarbhavi et al. 2006).

4.4 Clinical presentation

PTLD presents itself with fever, malaise, an infectious mononucleosis-like syndrome, lymphadenopathy and symptoms regarding organ dysfunction (Kalinova, Indrakova et al. 2009). Symptoms are often nonspecific, and some patients are asymptomatic. PTLD frequently presents as a rapidly enlarging mass in the grafted organ, in lymph nodes, filling the marrow space, or in extranodal sites such as upper airway or intestine. In young children, primary EBV infection often occurs after iatrogenic immunosuppression commences, either when an infected graft is introduced or later in the posttransplant period. Despite PTLD can present with symptoms reminiscent of infectious mononucleosis, but PTLD is a much more serious illness (Gulley and Tang 2010).

The risk of infection among allogeneic hematopoietic stem cell transplant recipients is determined by patient age, underlying disease, the complication that occurred during preceding treatment regimens, the selected transplantation modality, and the severity of graft-versus-host disease (GVHD) (Balletto and Mikulska 2015). In comparison with patients undergoing high-dose chemotherapy and autologous stem cell transplantation, aHSCT recipients are at a much higher risk of infection, due to delayed recovery of T-cell and B-cell functions (Ritter, Seitz et al. 2015). Immunological reconstitution after hematopoietic retrieval has an impact on the type of posttransplant infectious complications, and infection-related mortality is significantly higher post-engraftment than during the short posttransplant neutropenia. RIC regimens have a lower risk of severe and deadly infections in the early posttransplant period (Safdar 2011). Indeed, the most critical risk factor is the drug regimen used to prepare the patient for transplant as well as the ongoing immunosuppressive drugs used to prevent graft rejection, for example, anti-thymocyte globulin (ATG) depletes T cells and therefore protects from graft rejection, but its increases the probability of PTLD occurrence (Landgren, Gilbert et al. 2009, Atilla, Atilla et al. 2017). Fludarabine, azathioprine, and other agents responsible for T cell suppression or mutagenicity are also associated with PTLD pathogenesis (Martinez and de Gruijl 2008, Landgren, Gilbert et al. 2009). In addition, patients having multiple rejections and consequently more interventions to amplify immunosuppression may be at higher risk of PTLD. Some HLA types influence identification of cells expressing external viral proteins, which influences the pathogenesis of EBV-driven lymphoproliferation (Gulley and Tang 2010).

4.5 Histologic interpretation and Classification

Due to its heterogenous profile, PTLD diagnosis and histological classification requires a tissue biopsy (Kalinova, Indrakova et al. 2009). This histological evaluation has implications on treatment decisions according to whether the target antigen for rituximab (CD20) is expressed (Parker, Bowles et al. 2010, Glotz, Chapman et al. 2012).

PTLD is divided into four major histopathologic subtypes with corresponding clinical and biologic features, as described in the *World Health Organization* sub-classification scheme: early lesions, polymorphic PTLD, monomorphic PTLD and classical Hodgkin lymphoma-type PTLD (Spasojevic-Dimitrijeva, Peco-Antic et al. 2014). There is no staging system for PTLD, since it is almost impossible to elaborate a scheme for a disease with such broad histological spectrum (Bowden, Ljungman et al. 2010).

Early lesions, occur within one year after transplant, are sub-divided into two groups: plasmocytic hyperplasia (PP) and infectious mononucleosis-like lesions. These lesions are

B cell derived polyclonal lesions and are characterized by preserving the affected tissue architecture (Bowden, Ljungman et al. 2010, Ibrahim and Naresh 2012). These type of lesions frequently involve tonsils, adenoids or lymph nodes (Ibrahim and Naresh 2012).

Polymorphic PTLD is a mixture of small to large lymphocytes and immunoblasts, with a presentation very similar to HL, with Reed-Sternberg-like cells (Ibrahim and Naresh 2012). This subtype includes EBV-infected neoplastic B cells as well as reactive CD4+ and CD8+ T cells and clonality assays reveal monoclonal B cells (Gulley and Tang 2008). Reducing the level of immunosuppression is frequently effective in reversing cell growth (Bakker, van Imhoff et al. 2007).

Monomorphic PTLD is the most common form of PTLD and can be divided in two types: B-cell and T-cell/NK-cell (Cai, Chen et al. 2015). Most cases of B-cell monomorphic PTLD are of a non-germinal center type, especially those who are EBV positive (~50% of all cases) (Choquet 2016). Occasional EBV-negative cases are more likely to occur later after transplantation (beyond 1 year) (Swerdlow 2007). Monomorphic T-cell/NK-cell PTLDs are rare, accounting for ~10% of all PTLDs, and are associated with peripheral T-cell lymphoma (Choquet 2016). Conventional lymphoma therapy is not necessarily needed, since monomorphic PTLD can be treated by reducing immunosuppression (Green 2001, Knight, Tsodikov et al. 2009).

Classical Hodgkin Lymphoma-Type PTLD is very similar to HL, although it has the Hodgkin-like form of polymorphic PTLD, and is always EBV positive (Pitman, Huang et al. 2006). As for monomorphic and polymorphic lesions, classic Hodgkin lymphoma-like PTLD is characterized by destroyed tissue architecture (Choquet 2016). This type of PTLD occurs late after transplant (after the first year) and response to therapy is generally favorable (Gulley and Tang 2010).

4.6 Treatment and Prognosis

The first line of treatment comprises the reduction or withdrawal of immunosuppression, while chemotherapy and radiation are applied as a second line treatment (Reshef, Vardhanabhuti et al. 2011). Other approaches include, surgery, antiviral therapy, anti-B-cell monoclonal antibody (rituximab) and cytotoxic T cells are currently being investigated (Kalinova, Indrakova et al. 2009, Kim, Kim et al. 2010, Parker, Bowles et al. 2010, Glotz, Chapman et al. 2012).

Immunosuppression after transplantation, in a patient who is a carrier of Epstein-Barr virus, seems to reduce the activity of the patient's EBV-specific cytotoxic T-cell surveillance, which

increases the chances of uncontrolled proliferation of EBV-infected B-cells and subsequent progression to PTLD (Allen, Preiksaitis et al. 2009). Moreover, transplant recipients experiencing primary EBV infection, during the early post-transplant period, seem to be particularly susceptible to developing EBV-specific PTLD of B-cell origin, mainly by lacking preexisting EBV-specific T-cell immunity (Allen, Preiksaitis et al. 2009, Glotz, Chapman et al. 2012).

PTLD almost always develops rapidly to a fatal outcome unless it is diagnosed and treated (Opelz and Dohler 2004). The ability to reduce or eliminate immunosuppressive drugs is a helpful strategy for restoring natural antiviral and antineoplastic immunity. Almost all types are primarily of B-cell origin. Over 90% of PTLD cases are associated with EBV infection. Immunosuppressive treatment leads to T-lymphocyte dysfunction which allows uncontrolled proliferation of EBV.

Histology has a strong prognostic significance in PTLD (prognosis is much worse in NK-, T-cell, and plasmablastic B-cell PTLD compared with polymorphic, DLBCL-type and Burkitt/Burkitt-like B-cell PTLD, and is more satisfactory in early lesion and plasmacytoma-like PTLD (Allen, Preiksaitis et al. 2009). Thus, the WHO histologic classification provides important information on the probable progression and outcome of the disease and thereby influence choices of treatment (Glotz, Chapman et al. 2012). EBV association may also impact on prognosis, as EBV-associated PTLD may have a better prognosis than EBV-negative PTLD and may need less chemotherapy. EBV antigen expression (EBNA-1, -2, -3, LMP etc.) may also help understanding PTLD response to a reduction in immunosuppression and thus help to select treatment (Shimoyama, Asano et al. 2009).

PTLD is associated with mortality rates reaching up to 70-90% in HSCT patients, and 5-year survival rates of 59%, although recent data suggest that outcomes have improve (Opelz and Dohler 2004, Al-Mansour, Nelson et al. 2013, Caillard, Porcher et al. 2013).

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5. Epstein-Barr Virus

5.1 Structure and Genome

The Epstein-Barr virus (EBV), also known as Human Herpesvirus 4 (HHV-4) belongs to the Herpesviridae family, gamma subfamily, and is the only known human *Lymphocryptovirus* (Kwok 2007, Grywalska, Markowicz et al. 2013). Like other members of the Herpesviridae, EBV virions have double-stranded, linear DNA genome, with approximately 172 kb pairs in length, encoding approximately 100 genes surrounded by a protein capsid (Young, Arrand et al. 2007). EBV has a series of 0.5 kb terminal direct repeats and internal repeat sequences, that divide the genome into short and long, large unique sequence domains (Arvin, Campadelli-Fiume et al. 2007). The EBV genome is linear in virus particles and circularized in infected cells (Kwok 2007, Odumade, Hogquist et al. 2011). EBV has a toroid-shaped protein core that is wrapped with DNA, a nucleocapsid with 162 capsomeres, a protein tegument between the nucleocapsid and the envelope, and an outer envelope with external glycoprotein spikes. These glycoproteins are essential in cell tropism, host range and receptor recognition. Mature virions have approximately 120-180 nm in diameter.

5.2 Epidemiology and Disease

EBV infections are most prevalent in developing countries, in populations of low socioeconomic status. In countries with proper hygiene practices, EBV seroprevalence tends to increase gradually with age, showing two seroconversions peaks: at 2 to 4 years and at 14 to 18 years. The mean seroprevalence in children is approximately 50% and increases steadily to a value of 90% to 99% in adults (Walling, Ray et al. 2007, Grywalska, Markowicz et al. 2013). EBV by itself, accounts for 0.5%-2% of all cancers, varying by geographic locations (Jha, Pei et al. 2016).

Geographical distribution of EBV is variable depending on genotypes. EBV strains are characterized as type 1 (B95.8-like) or type 2 (Jijoye and AG876-like) (originally referred as A and B, respectively) differing in organization of the genes that encode the EBV nuclear antigen (O'Mahony, Debnath et al.) The two major types, 1 and 2, which are distinguished by genomic difference in a subset of latent genes, who encode nuclear proteins in latently infected cells (EBNA-LP, EBNA2, EBNA3A, EBNA3B and EBNA3C) (Dolan, Addison et al. 2006, Janani, Malathi et al. 2015). Both types are detected all over the world, with type 1 being more frequent in the Western hemisphere and Southeast Asia (Sample, Young et al. 1990, Odumade, Hogquist et al. 2011). Although, in central Africa, Papua New Guinea and Alaska, type 2 is more prevalent (Hjalgrim, Friborg et al. 2007). EBV type 1 is frequently

found in healthy individuals, while type 2 is associated with immunocompromised patients (Janani, Malathi et al. 2015).

There is no consistent data according to EBV seroprevalence by sex in children, although, in developed countries, where infection occurs in the adolescence, a higher seroprevalence and earlier occurrence of infectious mononucleosis is observed in women (Crawford, Swerdlow et al. 2002). Recent studies showed that an individual can harbor multiple viral strains, and these strains might be acquired during primary infection (Kwok 2007, Atalay, Gokahmetoglu et al. 2014) Arvin, Campadelli-Fiume et al. 2007).

5.3 EBV Infection and Latency

Transmission

The virus is transmitted through saliva, spread through close human oral contact, or transmitted by transfusion, and is highly immunogenic (Grywalska, Markowicz et al. 2013). Infection by EBV has several clinical displays since children are often asymptomatic, and have self-limited brief viral illness, whereas in adults it appears as infectious mononucleosis, in 30 to 50% of cases (Rea, Russo et al. 2001, Pittaluga 2013, Atalay, Gokahmetoglu et al. 2014).

Primary Infection

The receptor necessary for viral entry on epithelial cells and B lymphocytes is the CD21 molecule, which was previously known as CR2. When *in vitro*, the infection of B lymphocytes leads to continuous cell proliferation resulting in immortalization. Moreover, when infecting epithelial cells, occurs an active replication, leading to virions production and host cell apoptosis (Figure 8). Viral replication happens in the cell of the oropharynx and shedding of the virus is maintained throughout the host life. Primary infection induces both humoral and cellular immune response, with production of antibodies against lytic and latent genes, as well for the glycoprotein 350 preventing the binding of CD21 on B cells (Eligio, Delia et al. 2010).

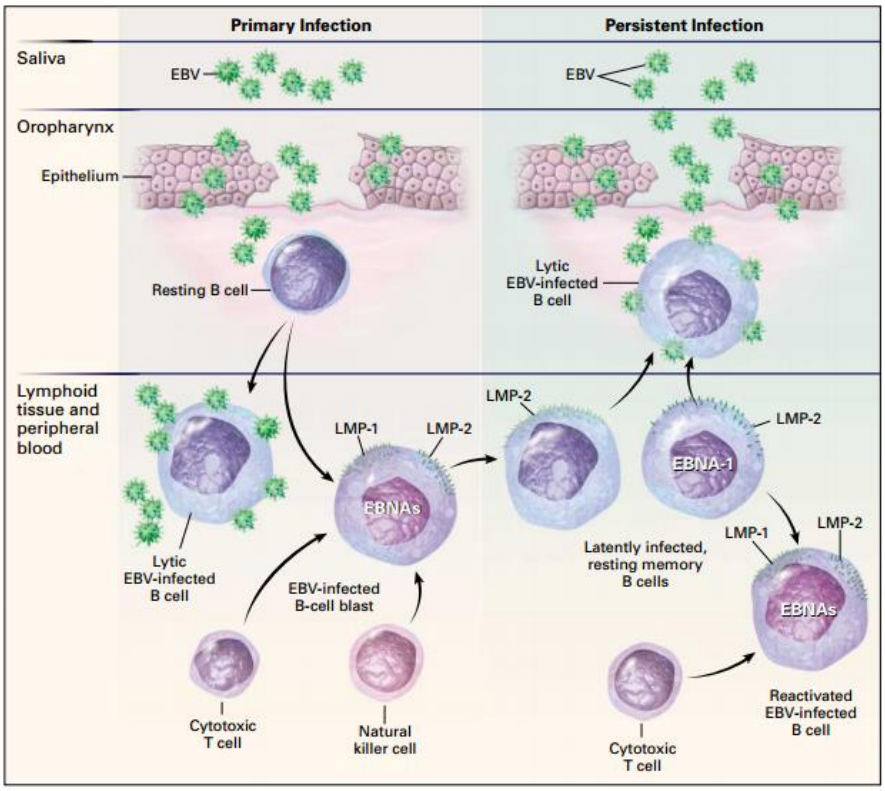

Figure 8 : Model of EBV infection in humans (Cohen 2000).

At primary infection, EBV produces a severe immune response against lytic antigens-mediated predominantly by CD8 and CD4 positive T cells (Pittaluga 2013). Once the infection occurs, the viral genome is maintained permanently in a small amount of B lymphocytes. Intermittent reactivation of the virus occurs in the oral mucosa, where shedding of virions in saliva spread the infection among human hosts. EBV can infect B lymphocytes as well as malignant cells of several lineages, including T lymphocytes, epithelial cells and smooth muscle cells (Gulley and Tang 2010, Chen 2011). EBV infection leads to B cell proliferation *in vitro* to form immortalized cell lines and also *in vivo*, when immune surveillance is diminished (Martinez and de Gruijl 2008, Gulley and Tang 2010). It is recognized that in the immunocompromised hosts, the interaction between EBV replication, latency and immune control can be disrupted and leads to prolonged proliferation of EBV-infected lymphocytes and their malignant transformation (Filipovich, Mathur et al. 1992). The virus is well adapted to normal B cell life cycle and has several different patterns of latent gene expression to establish and maintain persistent infection (Grywalska, Markowicz et al. 2013). Systematic reactivation of an infection is generally kept in check by the healthy immune system that inhibits lytic replication using cytotoxic T lymphocytes, natural kill cells and antibody-dependent cell cytotoxicity (Gulley and Tang 2010).

Latency

EBV latency comprises five EBV-encoded nuclear antigens (EBNAs), two latent membrane proteins (LMPs), EBV-encoded small RNA (EBER) and non-transcribed BART RNAs. EBERs are present in high amounts of copies in latently infected cells, therefore, these transcripts are targeted by *in situ* hybridization (ISH) on tissue sections.

EBV infection establishes four different latency patterns depending on which viral proteins are expressed. Latency 0 is defined by non-expression of viral proteins, where viral gene expression is limited two small non-coding, non-polyadenylated RNAs (EBER 1/2) and a set of transcripts from BamA rightward transcript (BART). This type of latency allows EBV to be persistence and become immunologically undetectable (Toczyski, Matera et al. 1994, Pittaluga 2013). Burkitt's lymphoma is associated with type I latency and expresses EBER1/2 RNA, EBNA-1, LMP-2A/B and BART RNA. Type II latency is associated with nasopharyngeal carcinoma and Hodgkin lymphoma, where EBV expresses EBER1/2 RNA, EBNA-1, LMP-1, LMP-2A/B (Carbone, Gloghini et al. 2008). Type III latency is when unlimited expression of all viral proteins are expressed: EBER 1/2 RNA, EBNA-LP, EBNA-1, EBNA-2, EBNA-3A/B/C, LMP-1, LMP-2A/B and BART RNA. This latency pattern occurs mainly in immunocompromised patients with post-transplant lymphoproliferative disorders, infectious mononucleosis, HIV-associated lymphoproliferative disorders and in lymphoblastoid cell lines (Grywalska, Markowicz et al. 2013, Kang and Kieff 2015).

Considering latently infected cells, a limited pattern of viral gene expression is observed: EBNA1 is required for maintenance of the episomal form of the virus, while EBNA2, the main transactivator protein of EBV, transactivates viral and cellular genes promoters. EBNA3 modulates the transactivator activity of EBNA2. The transmembrane proteins LMP1 and LMP2 affect several signal transduction pathways. The EBERs and BARTs, and the BARF1 protein are also associated with oncogenesis. The microRNAs derived from transcripts of the BHRF1 gene and the BART transcripts target both viral and cellular RNAs affecting the quantity (BALF5) and latent (LMP1) EBV transcripts as well as certain cellular RNAs. When EBNA2 is absent (the main viral transactivator protein in latency type III), the LMP promoters are triggered by cellular proteins in nasopharyngeal carcinomas and in Sternberg-Reed cells of Hodgkin's disease (latency type II), but not in type I BL cell lines or BL biopsies (type I latency) or memory B cells (type 0 latency) (Takacs, Segesdi et al. 2009).

Lytic Infection

EBV encoded proteins are necessary in productive infection and include transactivators, which are enzymes necessary for viral DNA amplification and assembly of structural compounds of the virions (Takacs, Segesdi et al. 2009). In the lytic cycle a progressive

cascade of gene activation is initiated: immediate early genes (BZLF1 and BRLF1) are expressed first and activate the early genes. The shift between latent and lytic infection is mediated by immediate early proteins (EB1 and Rta). These proteins are transcriptions factors that activate EBV early genes, which are necessary for viral DNA replication. Late proteins, who are only expressed after viral DNA synthesis, encode proteins necessary for assembly, maturation and release of infectious particles (Aubry, Mure et al. 2014).

5.4. EBV and Disease

EBV was the first human virus to be directly implicated in carcinogenesis (Grywalska, Markowicz et al. 2013). EBV was first discovered in 1964 by Epstein, Achong and Barr, from culture tumor cells, as the agent responsible for Burkitt's lymphoma in East African biopsy samples (Epstein 2001, Kwok 2007, Stanfield and Luftig 2017). Several studies have also demonstrated the ability of the virus to transform human B cells into lymphoblastoid cell line, suggesting its oncogenic potential (Stanfield and Luftig 2017).

Since its discovery, EBV has been found in a variety of other tumor types. The evidence for an association with EBV is strongest for Burkitt's lymphoma, NK/T cell lymphoma, nasopharyngeal carcinoma, Hodgkin's lymphoma and for malignant lymphomas in immune incompetent patients (Figure 9). Additionally, certain epithelial cell tumors, such as nasopharyngeal carcinoma and more recently to gastric carcinoma have been found to be EBV related (Gulley and Tang 2010, Grywalska, Markowicz et al. 2013, Sousa, Breda et al. 2013, Sousa, Mesquita et al. 2016).

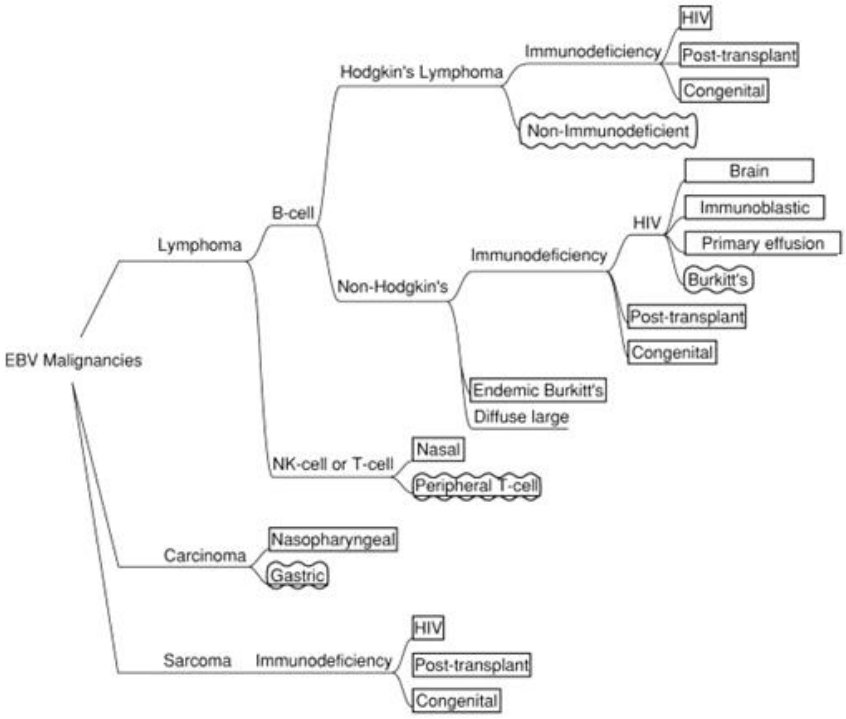

Figure 9: EBV-associated malignancies (Ambinder and Cesarman 2007).

5.5 Epstein-Barr Virus and PTLD

In post-transplant patients, immune surveillance is diminished by iatrogenic immunosuppression, which may lead to uncontrolled proliferation of EBV, leading to the development of PTLDs (Savoie, Perpete et al. 1994, Allen, Alfieri et al. 2002, Gulley and Tang 2010). Overall, 60 to 80% of all PTLDs are EBV-related, and the incidence of EBV varies according to type of PTLD (Petrara, Giunco et al. 2015). Type III latency is exhibited by the EBV-positive B cells in PTLD (Brink, Dukers et al. 1997). The wide expression of the latent EBV encoded proteins suggests an important role that EBV play in the oncogenic process. The mechanism by which EBV is thought to contribute to the pathogenesis of PTLD is similar to its presumed role in HL (Grywalska, Markowicz et al. 2013).

Tumorigenesis depends on several factors and the continuous presence pf the EBV within the lymphoproliferative region suggests that EBV is part of the process. EBV has both latent and lytic infection. In the lytic infection, EBV causes apoptosis of the infected cells, thus the tumors require the expression of latent programs to survive. LMP-1 is the major oncogenic protein of EBV and is essential for EBV-driven tumorigenesis (Young and Murray 2003). This protein acts like CD40, a member of the tumor necrosis factor receptor (TNFR), LMP-1 leads to the expression of anti-apoptotic proteins, such as BCL-2 and A20, and cytokines,

such as IL-1 and CD40L. EBV malignancies require the induction of telomerase activity, which is a ribonucleoprotein complex, that extends the telomeres at the ends, preventing cell senescence and apoptosis (Petrara, Giunco et al. 2015).

Analogous EBV-driven lymphoproliferations are observed in other immunosuppressive pathologies, such as chemotherapy-related immunosuppression for autoimmune disease or age-related decline in immunity, suggesting that reduced immunity is another major tumorigenic cofactor (Martinez and de Gruijl 2008, Gulley and Tang 2010). Diminished T cell immunity leads to uncontrolled proliferation, which can develop rapidly and systemically if not rapidly identified and treated (Gulley and Tang 2010). Although immunosuppression is a major factor in PTLD development, also persistent immune activation and chronic inflammation are important factors. Chronic inflammation leads to overproduction of B-cell stimulatory cytokines, such as IL-6, IL-10, IFN-α and TNF, thus enabling EBV proliferation (Petrara, Giunco et al. 2015).

Considering that most cases of PTLD are from donor or recipient derived B-cells, one approach to avoid the development of PTLD is to eliminate EBV infected B-cells in the early phase after aHSCT. Elevated levels of EBV DNA are present in blood samples of infected patients, including intracellular EBV within circulating B lymphocytes and extracellular EBV DNA measurable in plasma. EBV viral load, as measured by quantitative molecular analysis of the viral genome, serves as biomarker for predicting and monitoring the course of PTLD (Gulley and Tang 2008, Kalinova, Indrakova et al. 2009, Gulley and Tang 2010). Indeed, monitoring the EBV viral load, in peripheral blood, in addition to administering preemptive rituximab therapy has recently been reported to be a successful approach to preventing the development of B cell PTLD (Alexander, Jarrett et al. 2000, Adhikary, Behrends et al. 2006, Tanaka, Takizawa et al. 2014). Furthermore, reduced anti-EBV nuclear antigen antibody levels have also been linked with an increased risk for PTLD, suggesting that monitoring these antibodies may also be a biomarker of PTLD development (Riddler, Breinig et al. 1994).

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OBJECTIVES

MAIN OBECTIVE

The aim of this study is to characterize EBV-associated PTLDs and evaluate EBV monitoring, as well as identifying genetic susceptibility for EBV infection and PTLD in aHSCT patients. The characterization of EBV-PTLD profile will allow determining genetic/viral markers that contribute for a better clinical approach to aHSCT patients.

SPECIFIC OBJECTIVES

The specific objectives of this study were developed according to three different studies:

- Report PTLD in aHSCT patients (study 1)
- Evaluate EBV infection in a cohort of aHSCT patients (study 2)
- Genetic susceptibility for EBV infection as a marker for PTLD risk (study 3)

STUDY I

POST-TRANSPLANT LYMPHOPROLIFERATIVE DISORDER IN HEMATOPOIETIC STEM CELL TRANSPLANT PATIENTS: A SINGLE CENTER RETROSPECTIVE STUDY BETWEEN 2005 AND 2012

Article published on Molecular Medicine Reports (Attachment I)

TITLE

POST-TRANSPLANT LYMPHOPROLIFERATIVE DISORDER IN HEMATOPOIETIC STEM CELL TRANSPLANT PATIENTS: A SINGLE CENTER RETROSPECTIVE STUDY BETWEEN 2005 AND 2012

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ABSTRACT

Background: Post-transplant lymphoproliferative disorder (PTLD), despite its rarity, is an important mortality/morbidity event in transplant patients. The purpose of the present study was to retrospectively examine the clinical and pathologic characteristics, and outcomes of PTLD at the Portuguese Oncology Institute of Porto.

Material and Methods: A retrospective review of patient information was performed for patients that developed PTLD following allogeneic hematopoietic stem cell transplant (aHSCT) and were diagnosed between 2005 and 2012. The present study included a total of 15 patients, 8 females (53.3%) and 7 males (46.7%), with different clinicopathological characteristics.

Results: The most frequent clinical condition inducing aHSCT was acute lymphocytic leukemia (40.0%). Conditioning regimens consisted primarily in busulfan and cyclophosphamide, with anti-thymocyte globulin, and myeloablation was the preferential treatment. Epstein-Barr virus (EBV) was present in all patients with a median time of diagnosis following transplant of 75 days (range, 25-485 days) and a median viral load of 4.75 log10 copies/ml (range, 3.30-6.26 log10 copies/ml). PTLD diagnosis was mainly assessed by clinical findings, and histological confirmation was available for 5 patients: 3 monomorphic, 1 polymorphic and 1 with early lesions of PTLD.

Discussion/Conclusion: To the best of our knowledge, this is the first study to describe PTLD cases in HSCT patients in Portugal. The data reinforces the importance of performing EBV monitoring in high-risk patients, particularly those receiving a transplant from mismatch/unrelated donors, and those with myeloablative conditioning regimen including antithymocyte globulin. The results also suggested that EBV viral load may be significant for the prediction of PTLD development.

KEYWORDS: Post-Transplant Lymphoproliferative Disorder; Post-Transplant Lymphoproliferative Disease, Epstein-Barr Virus.

INTRODUCTION

The development of lymphoid proliferations after transplantation has been recognized for more than a quarter of century as an important morbidity factor (Castellano-Sanchez, Li et al. 2004). The post-transplant lymphoproliferative disorder (PTLD) refers to a heterogeneous group of lymphoproliferative diseases, which vary from uncomplicated, self-limiting infectious mononucleosis, to malignant lymphoma. The histological characterization varies from reactive-appearing, polyclonal lymphoid infiltrates or undifferentiated cells that are morphologically indistinguishable from malignant lymphoma or plasma cell myeloma (Gulley and Tang 2010, Mucha, Foroncewicz et al. 2010, Glotz, Chapman et al. 2012).

PTLD is relatively rare; nevertheless, it is the most frequent malignant disease early after transplantation, with the majority of cases being reported in the first year after transplantation (Kalinova, Indrakova et al. 2009, Glotz, Chapman et al. 2012, Atalay, Gokahmetoglu et al. 2014). Risk factors for PTLD development include young age and age over 50 years at transplantation, white race, unrelated or HLA-mismatched graft, Epstein-Barr virus (EBV)-seronegative status prior to transplant, primary EBV infection, type of organ transplant, intensity of immunosuppression and the occurrence of concomitant cytomegalovirus disease (Kim, Kim et al. 2010, Glotz, Chapman et al. 2012).

Not all PTLD cases are EBV-related, but consistent data recognize primary EBV infection as the most important risk factor for PTLD development (Funch, Walker et al. 2005, Caillard, Lelong et al. 2006). Indeed, the immunosuppression after transplantation in an EBV-seropositive patient reduces the activity of the patients' EBV-specific cytotoxic T-cell surveillance, which increases the probability of uncontrolled proliferation of EBV-infected B-cells and subsequent progression to PTLD (Allen, Preiksaitis et al. 2009). Moreover, transplant recipients experiencing primary EBV infection during the early posttransplant period seem to be particularly susceptible to developing EBV-specific PTLD of B-cell origin, reflecting their lack of any preexisting EBV-specific T-cell immunity (Allen, Preiksaitis et al. 2009, Glotz, Chapman et al. 2012).

The overall incidence of PTLD varies from 1 to 22% depending on the presence of risk factors, namely the transplanted organ, patient age, EBV serostatus from recipient and donor, aggressiveness of immunosuppression and combination of risk factors (Bar-Natan and Nagler 2006). The cumulative incidence of PTLD in allogeneic hematopoietic stem cell transplantation (HSCT) recipients is 1.0% (range 0.5-1.8%), with slightly higher rates in the pediatric population (Castellano-Sanchez, Li et al. 2004, Grywalska, Markowicz et al. 2013). Survival rates depend mainly on the type of PTLD, extent of disease and patient age: While pediatric patients and those with localized disease seem to have a better prognosis,

monomorphic PTLDs are the most aggressive forms (Kalinova, Indrakova et al. 2009, Kim, Kim et al. 2010, Luo, Zhang et al. 2014).

The purpose of this study was to examine the clinical and pathologic characteristics, as well as the outcome of PTLD after allogeneic hematopoietic stem cell transplant (aHSCT), in patients diagnosed at the Portuguese Oncology Institute (Porto, Portugal) between 2005 and 2012.

MATERIALS AND METHODS

Type of study and study participant

We retrospectively reviewed the information of patients that developed PTLD after aHSCT at the Portuguese Oncology Institute of Porto (IPO Porto) between 2005 and 2012. The study included a total of 15 patients, 8 females (53.3%) and 7 males (46.7%), with different lymphoid malignancies who attended at the Bone Marrow Transplant Service of IPO Porto. All cases included were histologically confirmed. PTLD diagnosis was performed by a Pathologist from the institution using to the World health organization (Spolverato, Kim et al.) Classification of Tumours of Haematopoietic and Lymphoid Tissues (4th Edition).

Sample processing and EBV detection

Samples were collected in EDTA-containing tubes (Vacutainer®; BD Biosciences, Franklin Lakes, NJ, USA) and stored in freezing temperature prior to processing. Blood samples were collected retrospectively from the institution archives. DNA was extracted by MagNA Pure Compact Nucleic Acid Isolation kit I (Roche Diagnostics GmbH, Mannheim, Germany). DNA/RNA quality was assessed by measuring the absorbance at 260/280 nm using the NanoDrop 1000 Spectrophotometer v3.7 (Thermo Fisher Scientific, Inc., Wilmington, DE, USA).

All patients submitted to aHSCT were monitored for EBV infection upon request from clinicians after clinical suspicion. EBV detection was performed at the Virology Service of IPO Porto using the commercial Real-Time PCR kit EBV Q-PCR Alert (Nanogen Advanced Diagnostics S.p.A., Trezzano sul Naviglio, Italy) which targets a region from EBV nuclear antigen 1 gene (EBNA1). Amplification was performed with the ABI PRISM 7300 Sequencer Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and results were obtained by measuring the geometric increase of probe fluorescence during amplification and samples were considered positive when the exponential curve exceeded the cycle threshold line. Regarding amplification quality, positive and negative controls were used: as negative control we used double distilled water in replacement of

template DNA; and as positive control we have used samples from the external quality control panel used at the Virology Service for EBV diagnosis.

Data collection

Clinic-pathological data was extracted from institutional databases including pre-transplant recipient age, gender, underlying disease, HLA-donor-recipient status, EBV serological status of the recipient, source of stem cells, conditioning regimen and use of ATG; post-transplant information (clinical findings, date of PTLD suspicion, date of PTLD confirmation, PTLD type, GVHD prophylaxis, GVHD type and outcome) and viral data (date of EBV suspicion, EBV viral load).

Statistical analysis

Statistical analysis was performed using the SPSS version 20.0 software (IBM Corp., Armonk, NY, USA). Overall survival was defined as the time between the date of transplant and the date of last follow-up or mortality. The differences in survival were calculated using the log-rank test and the Kaplan-Meier method.

RESULTS

The study included a total of 15 patients, 8 females (53.3%) and 7 males (46.7%), with median age of 10 years-old (range 3-38) - Table IA. Patients had a median follow-up time of 14 months (range: 2-72). Primary diagnoses of patients included in this study included paroxysmal nocturnal hemoglobinuria (n=1), primary immunodeficiency (n=1), acute lymphocytic leukemia (n=6), acute myelogenous leukemia (n=4), chronic myelogenous leukemia (n=1), myelodysplastic/myeloproliferative syndrome (n=1) and congenital amegakaryocytic thrombocytopenia (n=1). Most of patients had mismatched/unrelated donors (73.3%) and the collection of cells was mainly performed by peripheral blood stem cells (80.0%). Myeloablative conditioning was used in 14 patients and ATG in 12 patients. Transplant-related information for each patient is described in Table IIA.

Regarding the clinical presentation of patients, 2 presented with fever, 12 had increased liver enzymes, adenomegalies were observed in 2 patients and 12 patients had also increased lactate dehydrogenase. EBV serological status prior to transplantation were evaluated according to presence of IgM and IgG titers in plasma samples. Serological status was divided in three groups: susceptible (absence of IgM and IgG), active infection (presence of IgM and/or IgG) and finally, past infection (absence of IgM and presence of IgG).

The development of EBV infection was present in all of 15 patients, with a median time of diagnosis after transplant of 68 days (range 29-464 days), with 80% (n=12) of them detected <180 days after transplant, and with a median viral load of 4.75 log10 copies/ml (range 3.30-6.26 log10 copies/ml; Fig. 1A). PTLD diagnosis occurred approximately in the same period where EBV infection occurred (mean 135, median 75 days and range 25-485 days vs. mean 130 days, median 68 days and range 29-464 days, respectively). PTLD classification was available for only 5 patients and included monomorphic-type PTLD (n=3), polymorphic PTLD (n=1) and reactive plasmocytic hyperplasia (early lesions) (n=1) (Table IIIA; Fig. 2A). The remaining cases were not histological confirmed, and diagnosis was established by considering all clinical findings.

We observed graft-vs.-host disease (GVHD) in 13 patients (93.3%): 3 with acute GVHD (20.0%), 2 with chronic GVHD (13.3%) and 8 with both (53.3%). Considering the grade of acute GVHD, all patients with clinical information had a grade of II or higher. Regarding chronic GVHD, 3 patients had an evolution of acute-to-chronic, while 7 had a de novo chronic GVHD; two patients experienced extensive disease and 5 had only limited disease (Table IIA).

As for the overall outcome, 8 patients are still alive (53.3%), 5 with no signs of disease (33.3%) and 3 with evidence of disease (20.0%); and 7 patients have died (46.7%), 4 from complications associated with the transplant (26.7%) and 3 from progression of disease (20.0%) (Table IIA). A Kaplan-Meier plot was obtained by evaluating the cumulative survival of these patients, in months (Fig. 3A).

Table 1A: Clinical characteristics of patients.

	N (%)
Age, median (range) years old	10 (3-38)
Sex, *n* (%)	
Male	7 (46.7%)
Female	8 (53.3%)
Underlying disease, *n* (%)	
Acute leucemia	10 (66.6%)
Chronic leucemia	1 (6.7%)
Myelodysplastic/Myeloproliferative syndrome	1 (6.7%)
Others	3 (20.0%)
HLA donor, *n* (%)	
Match/Related	4 (26.7%)
Mismatched/Unrelated	11 (73.3%)
Source of cells, *n* (%)	
PBSC	12 (80.0%)
BM	2 (13.3%)
UCB	1 (6.7%)
Conditioning regímen, *n* (%)	
MAC	14 (93.3%)
RIC	1 (6.7%)
ATG, *n* (%)	
Yes	12 (85.7%)
No	2 (14.5%)

Legend: ATG: anti-thymocyte globulin; PBSC: peripheral blood stem cells; BM: bone marrow; UCB: umbilical cord blood; RIC: reduced-Intensity conditioning; MAC: myeloablative conditioning; HLA: Human leukocyte antigen.

Table 2A: Transplant-associated patient information.

Patient	Age (years)	Gender	Diagnosis	Pre-conditioning	ATG	Myeloablation	Donor	Source	GVHD prophylaxis	GVHD type	Outcome
1	25	Male	PNH	BuCy2ATG	Yes	Yes	UMD	PB	Tacrolimus	Acute	Mortality
2	6	Male	PI	Bu12Cy2ATG	Yes	Yes	UMD	BM	Tacrolimus+MTX	Acute	Alive
3	6	Male	ALL	Bu12Cy120MelfATG	Yes	Yes	UMD	PB	Tacrolimus+MTX	Acute/Chronic	Alive
4	38	Female	ALL	Bu12Cy120MelfATG	Yes	Yes	UMD	PB	CSP+MTX	Chronic	Alive
5	16	Male	ALL	Bu12Cy120MelfATG	Yes	Yes	UMD	PB	Tacrolimus+MTX	Acute/ Chronic	Mortality
6	23	Female	ALL	BuCyMelfATG	Yes	Yes	UMD	PB	Tacrolimus+MTX	Acute	Mortality
7	8	Male	ALL	BuCyMelfATG	Yes	Yes	UMD	PB	Tacrolimus+MTX	Acute/ Chronic	Mortality
8	28	Male	AML	BuCy2ATG	Yes	Yes	UMD	PB	Tacrolimus+MTX	Acute/ Chronic	Alive
9	10	Male	AML	Bu12Cy2ATG	Yes	Yes	UMD	PB	Tacrolimus+MTX	Chronic	Mortality
10	36	Female	AML	BuCy2ATG	Yes	Yes	MRD	PB	Tacrolimus+MTX	Acute/ Chronic	Alive
11	6	Female	AML	Bu16Cy4ATG	Yes	Yes	UMD	BM	Tacrolimus+MTX	Acute/Chronic	Alive
12	3	Female	CML	BuCyATG	Yes	Yes	UMD	UCB	Tacrolimus	NA	Alive
13	19	Female	MDS	BuCy2ATG	Yes	Yes	UMD	PB	Tacrolimus+MTX	Acute/ Chronic	Mortality
14	4	Female	CAT	AlentuzumabFluCy	Yes	Reduced Intensity	UMD	PB	T-cell depletion	NA	Mortality
15	8	Female	ALL	BuCyMelf	No	Yes	UMD	PB	Tacrolimus+MTX	Acute/ Chronic	Alive

PNH: paroxysmal nocturnal hemoglobinuria; PI: primary imunodeficiency; ALL: acute lymphocytic leukemia; AML: acute myelogenous leukemia; CML: chronic myelogenous leukemia; MDS: myelodysplastic/myeloproliferative syndrome; CAT: congenital amegakaryocytic thrombocytopenia; Bu: busulfan; Flu: fludarabine; Cy: cyclophosphamide; ATG: antithymocyte globulin; Melf: melphalan; PB: peripheral blood; BM: bone marrow; UCB: umbilical cord blood; MTX: methotrexate; CSP: cyclosporine; UMD: unrelated/mismatch donor; MRD: match/related donor; NA: not available; GVHD: graft-versus-host disease.

Table 3A: Characteristics of PTLD and EBV infection of patients.

Patient	Age (years)	Gender	Diagnosis	Clinical findings	EBV IgM	EBV IgG	EBV Serostatus	TT EBV infection (days)	Viral Load (copies/mL)	Viral load (Log copies/ml)	PTLD Classification	Biopsy vs. Excision (topography)
1	25	Male	PNH	Fever; Adenomegaly; Hepatomegaly; ↑ liver enzymes and LDH	NR	R	Past Infection	53	1,10E+07	5,64	NA	NA
2	6	Male	PI	Fever; ↑ liver enzymes	NR	R	Past Infection	25	4,40E+05	4,20	NA	NA
3	6	Male	ALL	↑ liver enzymes	NR	R	Past Infection	123	1,60E+04	5,76	NA	NA
4	38	Female	ALL	↑ liver enzymes and LDH	NR	R	Past Infection	38	5,70E+05	5,04	NA	NA
5	16	Male	ALL	↑ liver enzymes and LDH	R	R	Active infection	161	1,10E+05	6,26	Monomorphic (Case 2)	Biopsy (amygdala)
6	23	Female	ALL	↑ liver enzymes and LDH	NR	R	Past Infection	75	1,80E+06	4,53	Monomorphic (Case 3)	Biopsy (amygdala)
7	8	Male	ALL	↑ liver enzymes and LDH	NR	R	Past Infection	46	3,40E+04	5,72	NA	NA
8	28	Male	AML	Adenomegaly; Pancytopenia ↑ liver enzymes and LDH	NR	R	Past Infection	81	5,30E+05	4,75	Monomorphic (Case 1)	Excision (Cervical lymph node)
9	10	Male	AML	↑ liver enzymes	NR	R	Past Infection	65	5,60E+04	3,30	NA	NA
10	36	Female	AML	↑ LDH	NR	R	Past Infection	333	2,00E+03	3,26	Early lesions (Case 5)	Excision (Lymph node)
11	6	Female	AML	↑ liver enzymes and LDH	NR	R	Past Infection	280	1,80E+03	4,23	NA	NA
12	3	Female	CML	↑ liver enzymes and LDH	NR	R	Past Infection	485	1,70E+04	6,08	Polymorphic (Case 4)	Excision (Cervical lymph node)
13	19	Female	MDS	↑ LDH	NR	R	Past Infection	42	1,20E+06	3,63	NA	NA
14	4	Female	CAT	↑ LDH	NR	R	Past Infection	49	4,30E+03	5,64	NA	NA
15	8	Female	ALL	↑ liver enzymes and LDH	NR	NR	Susceptible	161	7,00E+03	3,85	NA	NA

PNH: paroxysmal nocturnal hemoglobinuria; PI: primary imunodeficiency; ALL: acute lymphocytic leukemia; AML: acute myelogenous leukemia; CML: chronic myelogenous leukemia; MDS: myelodysplastic/ myeloproliferative syndrome; CAT: congenital amegakaryocytic thrombocytopenia; LDH: lactate dehydrogenase; NR: non-reactive; R: reactive; TT: time to; NA: not available; EBV: Epstein-Barr virus; PTLD: post-transplant lymphoproliferative disorder.

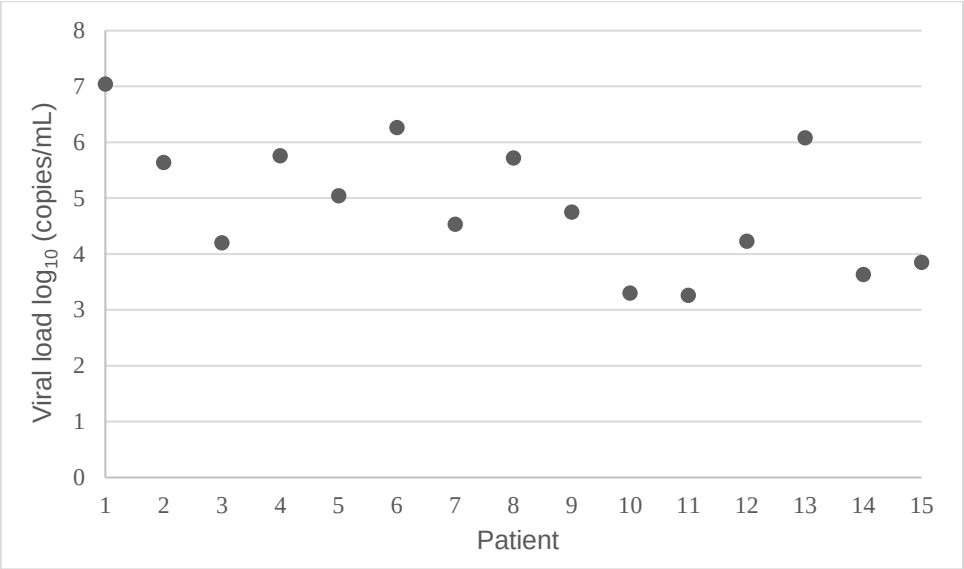

Figure 1A: Viral load distribution from all patients.

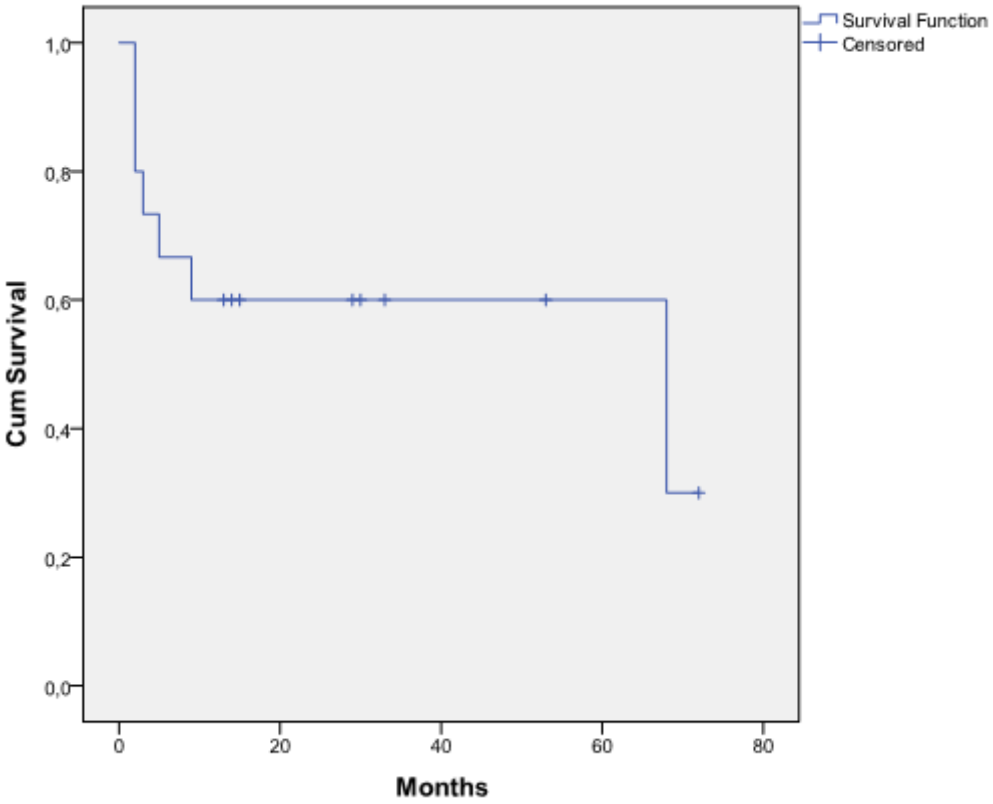

Figure 2A: Kaplan-Meier plot for survival analysis of PTLD patients.

DISCUSSION

PTLD is one of the most serious complications of immunosuppression in patients who undergo hematopoietic stem cell transplantation with high impact on morbidity and mortality in patients (Loren, Porter et al. 2003). EBV infection has been strongly associated with the development of PTLD, although, EBV-negative PTLD cases have been reported especially late after transplantation (>1 year) (Choi, Park et al. 2010, Ibrahim and Naresh 2012).

In this retrospective analysis, we verify that PTLD affects individuals of all age groups and several types of hematological malignancies and the majority have had unrelated donors. Our patients had different types of pre-conditioning regimens (myeloablative in 14 patients), with predominance in busulfan and cyclophosphamide. Since the types of regimen are varied, they appear not to have a direct correlation with the development of PTLD. ATG was used in almost all patients, except for one, and without absolute prevalence date it is difficult to confirm if its use is directly correlated with PTLD development. GVHD prophylaxis was performed mainly with tacrolimus, and concomitant with MTX, and still patients have developed some type of GVHD which indicates that altering prophylaxis regimen should be taken in consideration.

EBV infection is associated with the intermediate period after allogeneic stem cell transplantation, which means, it occurs mainly after 3 weeks to 3 months posttransplant Burns, Rana et al. (2016). In our case series, EBV infection was diagnosed at a median of 68 days after transplant. Viral infection during this period is correlated to delayed or incomplete reconstitution of specific immunity, or patients experiencing GVHD (Safdar 2011).

Frequently, the median onset of PTLD development is 3 months, with a range of 2-5 months after transplantation (Luo, Zhang et al. 2014), which is consistent with our data. Symptoms are quite nonspecific, with patients presenting with fever, malaise, enlarged lymph nodes and high levels of LDH, which were the factors for clinical PTLD suspicion in our patients (Gulley and Tang 2010). All patients that developed PTLD had an EBV infection at some point prior to transplantation. EBV positivity is directly related to PTLD development since its infection, or increase in viral load up to 2000 copies/mL, occurs mainly, at the same time PTLD is diagnosed. PTLD is more frequent in EBV-seronegative patients receiving allografts from EBV-seropositive donors and in patients with delayed immune reconstitution due to T-cell-depletion or HLA-mismatched donor. In a study conducted by Brunstein, Weisdorf et al. (2006), 15 of 335 patients developed a EBV-related complication, at a median of 133 days (range 52-407 days) which is consistent with our results (Brunstein, Weisdorf et al. 2006).

As previously described by Al-Mansour, Nelson et al. (2013), PTLD has mortality rates reaching up to 70-90%, which is higher than our results (46.7%) (Bhatia, Ramsay et al. 1996). Survival rates depend on age and stage of disease at the time of diagnosis, with pediatric and patients with localized disease with best prognosis (Kalinova, Indrakova et al. 2009). In our study, overall patient survival was not affected by the development of PTLD.

This study demonstrates that frequent monitorization of EBV viral load in patients receiving aHSCT is extremely important. The infection occurs mainly between 2 and 4 months after transplant and precedes the development of PTLD, and especially the viral load may be of significant importance for the monitorization and early diagnosis of PTLD.

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All authors declare no competing financial interests.

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STUDY II

ASSOCIATION OF EPSTEIN-BARR VIRUS INFECTION WITH ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION IN PATIENTS IN PORTUGAL

Article published on Molecular Medicine Reports (Attachment II)

TITLE

ASSOCIATION OF EPSTEIN-BARR VIRUS INFECTION WITH ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION IN PATIENTS IN PORTUGAL.

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ABSTRACT

Background: Epstein-Barr virus (EBV) infection in immunocompromised patients, such as hematopoietic stem cell transplanted patients can play a major role on the clinical outcome. The identification of patients at higher risk of developing EBV infection may be useful for the prevention of EBV-associated diseases.

Material and Methods: This prospective study was developed with 40 patients (27 male and 13 females, with mean age of 32.2±1.5 years old) randomly selected from the cohort of patients undergoing aHSCT at the Bone Marrow Transplant Service of IPO Porto. Between 1st January to 31st December 2015. EBV was tested in blood samples collected during routine procedures at Day 30, 60, 90, 120, 150 and 180 post-transplant.

Results: In our study we verified that 70.0% of our patients were EBV positive at least once during the follow-up period. Regarding the correlation of EBV infection and clinical features of patients, we verified that patients with unrelated donors had increased association with EBV infection at 60- and 150-days post-transplant (OR=3.9, *p*=0.058; OR=8.0, *p*=0.043; respectively). Moreover, myeloablative conditioning (OR=4.3, *p*=0.052), ATG use (OR=12.0, *p*=0.030) and GVHD (OR=6.7, *p*=0.032) were associated with EBV infection at at day 60, day 150 and day 90, respectively. Despite not statistically significant Overall Survival (OS) was associated with EBV infection at day 90 and 180 after transplant (*p*=0.095 and *p*=0.097, respectively). In our series, none patient developed PTLD disease.

Discussion/Conclusion: This is the first study to report the prevalence of EBV infection at different points in patients undergoing aHSCT from Portugal. Our study reveal that a high percentage of patients will develop an EBV infection during the post-transplant period and that some cofactors may influence its development. In our series EBV infection seems to be correlated with transplant from unrelated donors, the use of myeloablative conditioning, ATG and the development of GVHD. This study reinforces the importance of performing EBV monitoring for a better management of post-transplanted complications.

KEYWORDS: EBV, Epstein-Barr Virus; HSCT, Hematopoietic stem cell transplant; GVHD, Graft-versus-host disease.

INTRODUCTION

Epstein-Barr virus (EBV) is a ubiquitous Human Herpesvirus and infects 50% to 89% of children and remains latent, in memory B cells, of approximately 90% of adults (Styczynski, Tridello et al. 2016). Viral infections are known to be a major cause of morbidity and mortality in patients undergoing hematopoietic stem cell transplants (HSCT) and Herpesvirus are known to be among the most common viral infections in these patients (Fan, Jing et al. 2016). Moreover, monitoring of EBV DNA in peripheral blood is routinely performed in several transplant centers, since these patients have a higher-risk of developing EBV-related diseases (Janani, Malathi et al. 2015).

HSCT is an effective therapy in the treatment of hematological malignancies. Leukemias and lymphomas are a major part of worldwide cancers (Howlader, Noone et al. 2017). The regimens required for transplant produce profound immune deficiency in the early period after transplantation (Curtis, Travis et al. 1999). This iatrogenic suppression of T cell function in transplant recipients, leads to EBV proliferation in B cells (Janani, Malathi et al. 2015). Conditioning regimens include: myeloablative (MAC) and reduced intensity conditioning (RIC). MACs include concomitant or single use of alkylating agents, such as cyclophosphamide (Cy) and busulfan (Bu), while RICs are mainly performed with fludarabine (Flu) or low doses of total body irradiation (TBI) (Xuan, Huang et al. 2012, Juric, Ghimire et al. 2016). Choice of conditioning depends on patient's age, underlying disease, relevant comorbidities and type of donor. These regimens are related to several risks such as infections, graft-versus-host disease (GVHD), post-transplant lymphoproliferative disorder (PTLD) (Fan, Jing et al. 2016). GVHD occurs in approximately 40% to 90% of transplanted patients (Funke, Moreira et al. 2016). ATG seems to be effective in GVHD prophylaxis, and is related to reduced rates of relapses and infections in adults who undergo bone marrow (BM) or peripheral blood stem cells (PBSC) transplant (Storek, Mohty et al. 2015). In allogeneic HSCT selection of grafts donor consists in matched-related sibling, mismatched-related, matched-unrelated and mismatched-unrelated (Gratwohl, Baldomero et al. 2010, Barriga, Ramirez et al. 2012). The priority is to find a related donor, and for patients who lack such, they must find an unrelated donor. The probability of finding a matched unrelated donor is around 30% to 70%, depending on the frequency of the HLA genotype in the donor registries and patient's ethnicity (Juric, Ghimire et al. 2016).

Rates of mortality after HSCT often reach up to 50.0% (Fan, Jing et al. 2016). Some studies describe that intensive conditioning regimens are associated with reduction of tumor relapses, although it might simultaneously increase the transplant-related mortality rates, including the mortality of infections (Xuan, Huang et al. 2012).

This study aims to analyze patients' clinical characteristics at time of transplantation and evaluating risk factors related to poor outcomes.

MATERIALS AND METHODS

Type of study and study participant

A prospective follow-up study was performed with patients who underwent aHSCT at the *Bone Marrow Transplant Service* of *Portuguese Oncology Institute of Porto* (IPO Porto) between 1st January to 31st December of 2015 that were selected randomly. In this study, 40 patients were included, 13 females (32.5%) and 27 males (67.5%), within a 6 months follow-up. The study used the samples that were collected during routine procedures for viral monitoring at the Virology Service of IPO Porto. The study was approved by the local Ethical Committee and did not interfere with the routine procedures decided by clinicians

Sample processing:

Blood samples were selected at 6 different times: 30, 60, 90, 120, 150 and 180 days after transplant. Samples were collected in EDTA-containing tubes (BD Vacutainer®, NJ) and stored prior to processing and DNA was extracted by *MagNA Pure Compact Nucleic Acid Isolation kit I* (Roche, Germany). DNA/RNA quality was assessed by measuring the absorbance at 260/280 nm using the NanoDrop 1000 Spectrophotometer v3.7 (Thermo Scientific, Wilmington, DE, USA).

EBV detection

EBV detection was performed by a real-time PCR protocol targeting EBV polymerase gene (EBV POL) with forward and reverse as previously reported (Marinho-Dias and Sousa 2013). Amplification was performed with the ABI PRISM 7300 Sequencer Detection System (Applied Biosystems) and results were obtained by measuring the geometric increase of probe fluorescence during amplification and samples were considered positive when the exponential curve exceeded the cycle threshold line.

Quality Control

Regarding quality control, all real-time PCR amplifications used positive and negative controls: as negative control, we used double distilled water in replacement of template DNA; and as positive control we have used samples from the External Quality Control panel

for EBV used at the Virology Service. Results were independently analyzed by two of the authors and 10% of all samples were randomly selected and re-submitted to amplification to confirm the results.

RESULTS

Clinical characteristics

This study included randomly selected patients who underwent aHSCT at our institution between 1st January and 31st December of 2015. Table 1B demonstrates the characteristics of all patients.

This study included 40 patients, 27 males (67.5%) and 13 females (32.5%), with ages between 1 and 63 years-old (mean: 32.2 ± 1.5 years old) (Figure 1B). Patients had different hematological malignancies, including aplastic anemia (n=3), acute leukemia (n=23), chronic leukemia (n=2), non-Hodgkin lymphoma (n=1), multiple myeloma (n=1), myelodysplastic/myeloproliferative syndrome (n=7) and others, including primary immunodeficiency, myelofibrosis and severe combined immunodeficiency (n=3). Off the 40 patients submitted to aHSCT only one was being transplanted for the second time. When evaluating the donor-receptor relation, 21 patients had unrelated donors (52.5%) and the remaining 19 had related donors (47.5%). Regarding the HLA-match, only one patient received a graft from a HLA-mismatched donor. The source of cells for transplant was mainly from peripheral blood (82.0%), while the remaining were from bone marrow (13.0%) and umbilical cord blood (5.0%).

Myeloablative conditioning was applied to 24 (60.0%) of our patients, with busulfan and cyclophosphamide as well as ATG which was used in 14 of these patients. Reduced intensity regimens were used in 16 patients with 6 of them receiving ATG.

Prophylaxis for GVHD was performed for all patients (data not available for 2 patients). Acute GVHD was observed in 21 patients, all of them with grade 2 or higher, while Chronic GVHD was present in 5 patients, 4 with evolution from aGVHD and only one with *de novo* cGVHD.

Serological EBV and CMV status was collected from clinical records: regarding EBV, 3 patients were susceptible for primary infection, 36 had past-infection, and one patient had an active EBV infection; regarding CMV, 7 patients were susceptible of primary infection and the remaining had past-infection.

Table 1B: Clinical characteristics of patients.

Characteristics of patients	Value
Age, years, median (range)	32.2 (1-63)
Sex (*n*, %)	
Male	27 (67.5%)
Female	13 (32.5%)
Underlying disease	
Aplastic anemia	3 (7.5%)
Acute leukemia	23 (57.5%)
Chronic leukemia	2 (5.0%)
Non-Hodgkin lymphoma	1 (2.5%)
Multiple myeloma	1 (2.5%)
Myelodysplastic/Myeloproliferative syndrome	7 (17.5%)
Others	3 (7.5%)
Conditioning regimen	
BuCy	4 (10.0%)
BuCy2	20 (50.0%)
Cy	1 (2.5%)
FluBu	10 (25.0%)
FluCy	3 (7.5%)
FluMelf	1 (2.5%)
ATG	
Yes	20 (50.0%)
No	20 (50.0%)
Type of donor	
Related	19 (47.5%)
Mismatched/Unrelated	21 (52.5%)
Source of cells	
PBSC	33 (82.5%)
BM	5 (12.5%)
UCB	2 (5.0%)

Legend: Bu: Busulfan; Cy: Cyclophosphamide; Flu: Fludarabine; Melf: Melphalan; ATG: Anti-thymocyte globulin; PBSC: Peripheral blood stem cells; BM: Bone marrow; UCB: Umbilical cord blood.

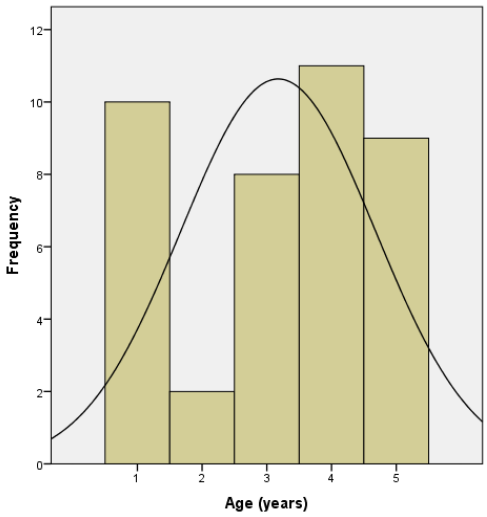


Figure 1B: Frequency of age groups (Group1 - <=11 years-old; Group 2 – 12-18 years-old; Group 3 – 19-36 years-old; Group 4 – 37-50 years-old; Group 5 – 51-63 years-old).

EBV infection analysis

Samples from all patients were collected at 6 periods post-transplant. Results regarding the detection of EBV demonstrated a prevalence of 38.3% positive samples and results according to periods are shown in figure 2B. Considering number of EBV positive samples we had: Day 30 - 10 samples (25.0%); Day 60 - 18 samples (47.4%); Day 90 – 13 samples (38.2%); Day 120 – 9 samples (31.0%); Day 150 – 10 samples (45.5%); Day 180 – 7 samples (58.3%).

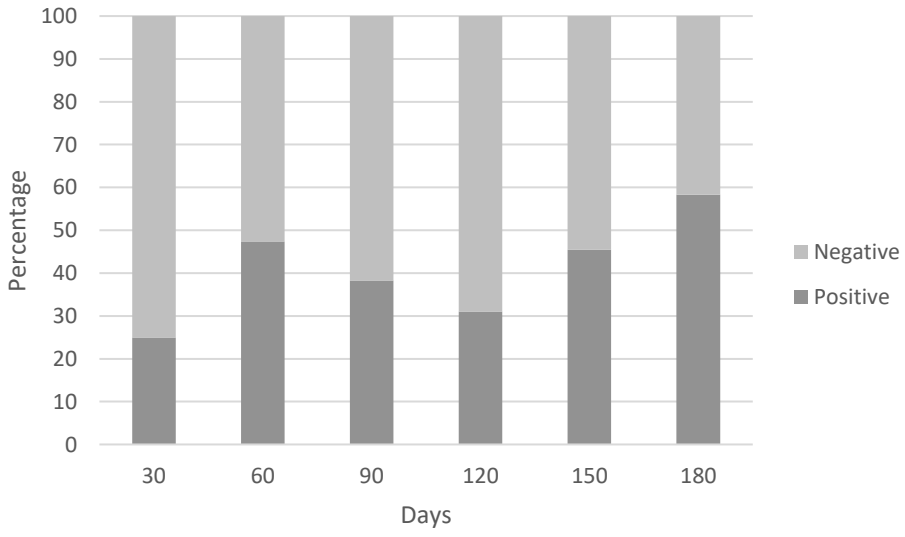


Figure 2B: Percentage of EBV infection at different stages post-transplant.

EBV infection and clinical characteristics

The analysis according to gender seem to show that post-transplant EBV infection is more frequent in females (OR=8.3, *p*=0.06), although with borderline statistical significance.

Despite not statistically significant, we have found that EBV infection is more frequent in patients with unrelated donors (OR=2.5, *p*=0.170), engrafted with PBSC (OR=2.0, *p*=0.410), patients submitted to myeloablative conditioning regimen (OR=2.4, *p*=0.166), using ATG in the conditioning regimen (OR=2.2, *p*=0.176), and that developed aGVHD (OR=2.5, *p*=0.170). EBV serostatus prior to transplant does not seem to be related with the development of infection during the post-transplant period. Patients with chronic GVHD were all EBV positive at least once, suggesting that cGVHD may also related with infection.

Additionally, we proceeded with the analysis of EBV infection at the different times during the follow-up period. At 60 days post-transplant, we verified that EBV infection was associated with transplants from unrelated donors OR=3.9, *p*=0.058), myeloablative conditioning (OR=4.3, *p*=0.052), and ATG use (OR=3.6, *p*=0.099). At 90 days only GVHD was related a higher risk of infection (OR=6.7, *p*=0.032). Finally, at 150 days, risk of infection was related with unrelated donors (OR=8.0, *p*=0.043), ATG (OR=12.0, *p*=0.03) and GVHD (OR=5.6, *p*=0.099).

By the analysis of cox regressions on cumulative survival considering patient's sex, donor, myeloablative/reduced intensity regimen, with/without ATG and development of aGVHD, we observed that there is an association of unrelated donor and EBV infection at 150 days post-transplant (HR=8.8, *p*=0.03).

Follow-up

Of the 40 patients here analyzed, we observed that 16 patients are currently deceased, 6 are alive with evidence of disease and 18 are alive and without evidence of disease. The cumulative survival was evaluated by performing a Kaplan-Meier plot (Figure 3B), and estimated survival time was approximately 476 days. Survival analysis was correlated with EBV DNA positivity and the following Kaplan-Meier plots were obtained (Figure 4a, b, c, d, e and f). Results showed that EBV DNA positivity at 90 days post-transplant, is associated with increased mortality (*p*=0.095), with EBV positive patients having approximately less 290 days than EBV negative patients (303.3 versus 593.2 days, respectively).

During this period, none of the patients developed PTLD, neither clinical signs suggesting the development of PTLD.

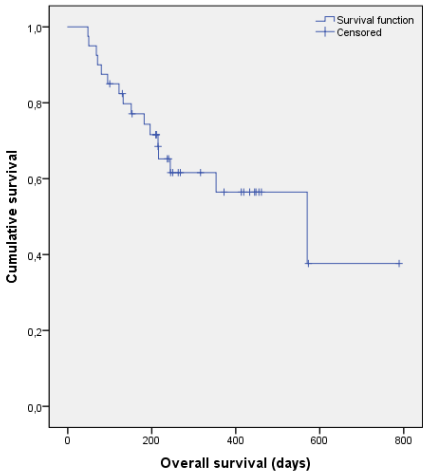
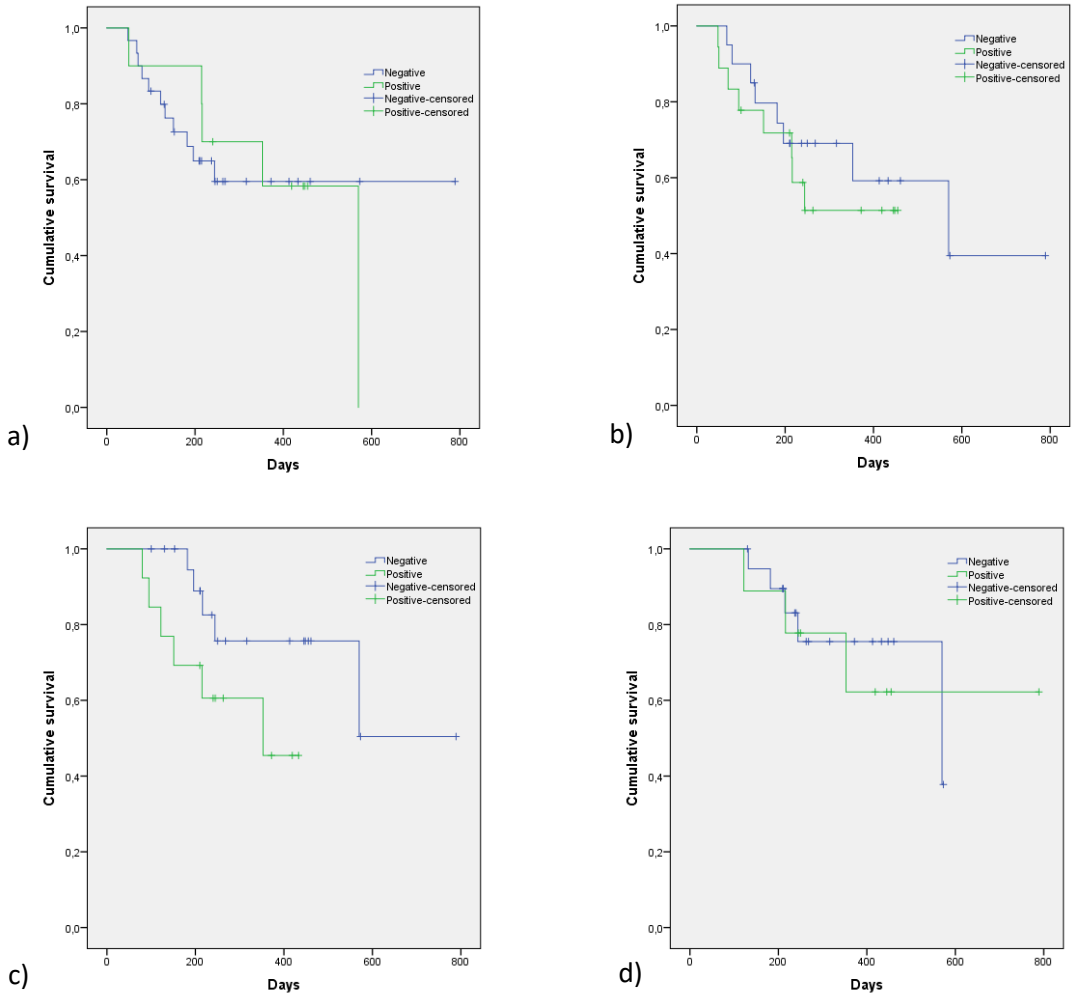
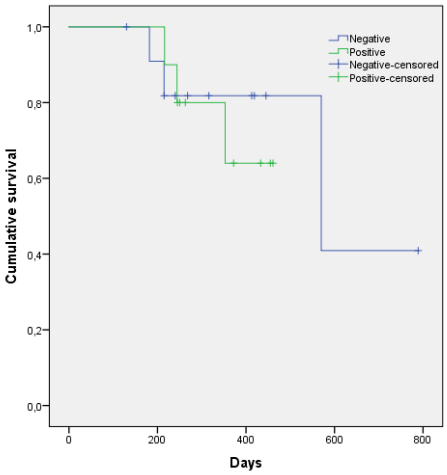


Figure 3B: Kaplan-Meier plot for survival analysis.



e)



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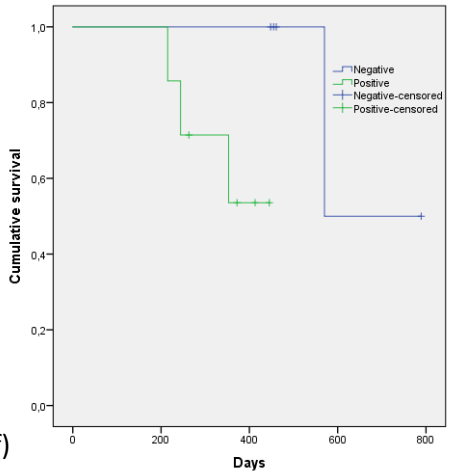

Figure 4B: Survival analysis when a) EBV positive +30 days post-transplant, b) EBV positive +60 days post-transplant, c) EBV positive +90 days post-transplant, d) EBV positive +120 days post-transplant, e) EBV positive +150 days post-transplant, f) EBV positive +180 days post-transplant.

DISCUSSION

Allogeneic hematological stem cell transplant (aHSCT) is an option for the treatment of hematological malignancies and these patients are submitted to pre-transplant treatments that reduce significantly the immune system to avoid rejection of the graft (Henig and Zuckerman 2014). This immunosuppression is associated with the occurrence of different events in the post-transplant period, such as development of graft-versus-host disease, infections and post-transplant lymphoproliferative disorders (Al-Mansour, Nelson et al. 2013).

Viral infections are a major concern in the subset of aHSCT, and while CMV infection has been consistently associated with a significant morbidity/mortality increase, EBV infection has been underestimated in these patients since only a minority will suffer from EBV-associated complications, such as PTLD (Preiksaitis 2004). Gulley et al., affirm that routine monitoring of EBV infection is viable in PTLD prevention, although further studies must be done to correlate specific viral loads to the identification of high-risk patients. Viral load is also informative, when there is a withdrawal of immunosuppression of rituximab administration, to verify if the treatment is successful (Gulley and Tang 2010).

In our study, we verified that 70.0% of our patients were EBV positive at least once during the follow-up period. Blaes et al., described that EBV viremia was present in 23.0% of their patients, which is very different from what we have found (Blaes, Cao et al. 2010). Esser et al., observed rates of EBV infection in 49.0% in patients submitted to SCT, once again lower than what we obtained (van Esser, van der Holt et al. 2001). Furthermore, EBV infection varied throughout the follow-up period, with a mean of 65.6±39.6 days (range 27-183). Duman et al., monitored EBV viral load at least once a week for 3 months and verified that EBV reactivation occurred in approximately 14.0% of patients, which is also much lower than what we describe (Dumas, Ruggeri et al. 2013). Styczynski et al., refer that screening of EBV DNAemia should start within the first month after aHSCT, and that monitoring should continue for at least 4 months after transplant, with a frequency of once a week (Styczynski, van der Velden et al. 2016). Despite this suggestion, many authors discuss the cost-effectiveness of EBV monitoring once-a-week and therefore many studies are required to show what would be the best time and interval of monitoring. Moreover, EBV viral load has been having different input data since the viral loads for treatment/follow-up cutoff are variable. Indeed, the development of international standards for EBV viral load management is yet to be defined (Marques, Shikanai-Yasuda et al. 2014).

Our data showed that patients with unrelated and/or mismatched donors were more prone to develop an EBV infection post-transplant, and that using peripheral blood as source of

stem cells adds a higher risk for EBV infection. Studies report EBV infections of 8.8% in myeloablative conditionings and 35.0% in reduced intensity conditionings (Cohen, Gandhi et al. 2005), 54.0% in T-cell depletion (van Esser, Niesters et al. 2002) and 65.0% in T-cell depletion concomitant with ATG use (van Esser, van der Holt et al. 2001). Despite UCB has been related with higher incidences of EBV viremia, many others have failed to show this (Dumas, Ruggeri et al. 2013). Nevertheless, studies with larger populations and preferably with equal proportions of PBSC, BM and UCB graft receptors, should be performed to clarify these evidences.

The intensity of conditioning regimen has been shown to directly affect the relapse and survival of patients who undergo aHSCT (Liu, Fan et al. 2009). In our study, myeloablation conditioning and use of ATG demonstrated a 3-times higher risk of developing EBV. These findings are according those described by Xuan et al. that observed that intensified conditioning increase the incidence of EBV viremia and disease. Furthermore, use of ATG, HLA-mismatched, unrelated donor and acute aGVHD were also identified as risk factors for EBV infection (Xuan, Huang et al. 2012).

When addressing the development of GVHD, we observed that patients who developed aGVHD, had 3-times increased association with EBV infection. Furthermore, we noticed that all patients with cGVHD, were positive for EBV at least once in the post-transplant follow-up. As described previously by Janeczko et al., GVHD is related to delayed immune reconstitution, favoring infections in the early period post-transplant. Moreover, viral infections are also associated with delayed IR and appear to be linked to the degree of immunosuppression. Therefore, monitoring of infections is critical in this phase (Janeczko, Mielcarek et al. 2016).

Analysis of EBV infection on different periods post-transplant revealed that unrelated donor (OR=3.9, p=0.058), myeloablation (OR=4.3, p=0.052) and the use of ATG (OR=3.6, p=0.099) seem to be risk factors for EBV infection occurrence at 60 days post-transplant; At 90+ days GVHD is connected to EBV infection (OR=6.7, p=0.032).; use of ATG (OR=12.0, p=0.030) , unrelated donor (OR=4.8, p=0.043) and GVHD (OR=3.3, p=0.099) are related to EBV infection at day 150 post-transplant.

Our mortality rates are from 40.0%, with a median follow-up period of 8.2±5.3 months (range 1-25 months). Similar findings have been reported by other authors, such as Styczynski et al., that reports a mortality rate of 45.2% in a follow-up period of 4.9 years (Styczynski, Tridello et al. 2016). EBV infection and its association with mortality were analyzed and data revealed that EBV DNA positivity at 90 days and 180 days post-transplant, are associated

with increased mortality (*p*=0.095, 303.3 *versus* 593.2 days and *p*=0.097, 367.*0 versus* 679.5).

At our institution, EBV detection is not performed routinely, and with these results, we demonstrate the necessity in EBV monitoring after transplantation, since infection seems to have a major role in complications after transplant and mortality. It is critical to develop a risk profile to determine which patients are at higher risk of developing EBV infection and further complications. This profile must be obtained by information on which risks factor are more related to EBV infection.

CONCLUSION

EBV infection is still a major concern in the subset of HSCT. Therefore, and with these results, monitoring and identification of which variables define high risk patients are necessary for preventing EBV-related complications and mortality.

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FINANCIAL DISCLOSURE

All authors declare no competing financial interests.

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STUDY III

SINGLE NUCLEOTIDE POLYMORPHISMS AS GENETIC SUSCEPTIBILITY MARKERS FOR EPSTEIN-BARR VIRUS INFECTION AND POS-TRANSPLANT LYMPHOPROLIFERATIVE DISORDER IN HEMATOPOIETIC STEM CELL RECIPIENTS

Article submitted for publication

TITLE

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ABSTRACT

Introduction: Epstein-Barr Virus (EBV) is one of the major risk factors for morbidity and mortality after allogeneic-Hematopoietic Stem Cell Transplantation (allo-HSCT). The identification of genetic polymorphisms in genes that regulate the immune response to viral infections may be useful in the definition of risk biomarkers for EBV-infection and/or the development of Post-transplant lymphoproliferative disorders (PTLD) in allo-HSCT recipients.

Material and Methods: A total of 39 patients who underwent allo-HSCT were studied. Single nucleotide polymorphism (SNPs) from HLA polymorphic sites and non-HLA regions including host immune response genes and antiviral effectors were selected and analyzed using MassARRAY iPLEX Gold technology. The analysis was performed in samples collected pre-and post-transplant and analyzed the risk of EBV infection and PTLD development.

Results: Overall, 31 patients (79.5%) were positive for EBV at least once in the follow-up period, and 9 patients were diagnosed with PTLD (2 with histological confirmation and 7 based on clinical findings. Pre- and post-transplant genotype analysis revealed that in the majority of SNPs more than 20% of patients had changed their genotype after transplant. Furthermore, we observed that IL-1B rs1143627 Acarriers (p=0.024; OR=14.0) are associated with EBV infection; and IL-1A rs2856838 AA genotype (p=0.049; OR=1.28), IL-10 rs3024496 GG genotype (p=0.037; OR=7.20) and MAVS rs6052130 Acarriers (p=0.009; OR 1.50) are associated with PTLD development.

Discussion: This is the first study analyzing the role of these SNPS in EBV infection and PTLD development in allo-HSCT recipients. The study identifies IL-1B rs1143627 (Acarriers) as a significant risk factor for EBV infection and IL-1A rs2856838 (AA genotype), IL-10 rs3024496 (GG genotype) and MAVS rs6052130 (Acarriers) as risk markers for PTLD development.

INTRODUCTION

Allogeneic hematopoietic stem cell transplant (allo-HSCT) is a potentially curative therapy for many haematological malignant disorders (Majhail et al., 2015). This procedure is often associated with a significant reduction of immune response and therefore after transplantation the development of opportunistic infections is considered a determinant factor for success. Indeed, viral infections are amongst the most frequent events after allo-HSCT, and are the leading causes of morbidity and mortality after transplant (Bollard and Heslop, 2016; Campos et al., 2017; Sousa et al., 2014).

Epstein-Barr virus (EBV) is one of the most important viruses in allo-HSCT recipients. Despite EBV is a ubiquitous virus, infecting around 90% of the population, which is associated with the development of several malignancies (Tan et al., 2018) including B-cell lymphoproliferative disorders, such as Burkitt and Hodgkin lymphoma, as well as post-transplant lymphoproliferative disorders (PTLD) (Mui et al., 2017). Patients undergoing allo-HSCT are at increased risk of EBV infection and consequently have an increased risk of developing post-transplant lymphoproliferative disorders (PTLDs) (Marinho-Dias et al., 2018a; Marinho-Dias et al., 2018b).

In the majority of population, the infection by EBV is often asymptomatic, and the virus transforms and immortalizes B lymphocytes (Hatton et al., 2014). In immunocompromised patients, such as those submitted to immunosuppressive therapy to avoid rejection or graft-versus-host disease (GVHD), T-cell function is inhibited which leads to uncontrolled proliferation of EBV-infected B-cells (Weikert and Blumberg, 2008). Genetic polymorphisms in genes that regulate the host immune response are of extreme importance for the identification of a genetic profile of viral infection susceptibility, associated with the initiation of an immune response to EBV infection, disease development, and prognosis (Akay et al., 2014; Johnson et al., 2015; Medina-Acosta et al., 2014; Song et al., 2017). Recent advances have been made to characterize the role of variations in the Major Histocompatibility Complex (MHC) (such as HLA-A, HLA-B and HLA-G from Class I; and HLA-DR and DQ from Class II) and genes that regulate the host immune response to viral infections (such as TNFA, IL-1a, IL-1b, IL-1RN, IL10, IFNG, IFNGRI and IFNGRII) and also genes involved in antiviral response (such as RIG-I, DDX58, MDA-5, IFIH1, MAVS, VISA, IPS1, CARDIF and ISG15 ubiquitin-like pathway) (Diepstra, Niens et al. 2005, McAulay, Higgins et al. 2007, Brennan and Burrows 2008, Hjalgrim, Rostgaard et al. 2010, Long, Taylor et al. 2011). Previous studies have shown that HLA-A*0101, A*0201, A*0207, A*1101, DRB1*07, DQA1*0103, DQA1*0201 are associated with susceptibility to EBV infection and development of certain associated diseases (Brennan and Burrows, 2008; Diepstra et al., 2005; Long et al., 2011; McAulay et al., 2007).

Although several studies have been made with the purpose of finding predictive factors for the development of EBV infection and PTLD development, there is no clear result that could be used in clinical management. The aim of this study is to characterize host genetic polymorphisms that could be used to predict the risk of EBV infection PTLD development in allo-HSCT.

MATERIAL AND METHODS

Type of study

We developed a hospital-based retrospective study with patients from the cohort of patients who underwent allogeneic hematopoietic stem cell transplant (aHSCT) at the *Bone Marrow Transplant Service* of *Portuguese Oncology Institute of Porto* (IPO Porto). Cases were selected according to sample availability and quality. The study (CI-IPOP 13/2014) was approved by the local Ethical Committee (CES 197/2014) and did not interfere with the routine procedures.

Population

Patients with different hematological malignancies who underwent aHSCT between February of 2011 and November of 2015. Inclusion criteria: pre-transplant serologic EBV characterization and follow-up after transplant according to the protocol for EBV monitoring. Clinicopathological data (age, gender, hematological diseases, EBV serological status, stem cell source, date of aHSCT, status of disease at the time of HSCT, GVHD, date of GVHD, complications, type of conditioning regimen and therapeutic approach/prophylaxis) was collected from individual clinical records and inserted on a database with unique codification.

Sample collection and processing

For each patient, two samples were selected: one at the time of transplant and one 4 months post-transplant. After transplantation, the reconstitution of different immune cells occurs at different time points (Ogonek, Kralj Juric et al. 2016) and Chimerism analysis is used for peri-transplant surveillance of engraftment (Bader, Niethammer et al. 2005). In our population this was used in all cases to confirm engraftment no later than 4 months after transplantation.

Peripheral blood samples collected following standard venipuncture techniques in EDTA-containing tubes were used for total nucleic acid (NA) extraction with High Pure Viral Nucleic Acid kit (Roche, Germany). Total NA were preserved at -80ºC and DNA/RNA integrity was analyzed using the NanoDrop® 1000 Spectrophotometer v3.7 (Thermo Scientific, Wilmington DE, USA).

Host genetic susceptibility profile characterization

Polymorphisms were selected from HLA polymorphic sites (class I, II and III) and non-HLA regions including host immune response genes (such as TNFA, IL-1a, IL-1b, IL-1RN, IL10, IFNG, IFNGRI and IFNGRII); antiviral effectors (such as RIG-I, DDX58, MDA-5, IFIH1, MAVS, VISA, IPS1, CARDIF and ISG15 ubiquitin-like pathway); and cellular glycoprotein (FUT2/3). SNPs were selected for study following biomedical literature search on PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) for SNPs associated with host immune response and antiviral effectors. SNPs from the selected genes were then elected according to the following criteria: 1) plausible functional mechanisms; 2) a minor allele frequency (MAF) of 10% or more in a Caucasian population according to the dbSNP database (http://ncbi.nlm.nih.gov/projects/SNP/); and 3) must represents a region of each selected gene, showing tight linkage disequilibrium (LD, r^2>0.8) with other SNPs throughout the selected gene, according to Hapmap database comparing data for our population (Caucasians residents of European ancestry from Utah - CEU).

SNP genotyping was performed using MassARRAY iPLEX Gold technology (Sequenom, SanDiego, CA), based on multi-plexed amplification followed by mass-spectrometric product separation. This technique was carried-out by the Genomic Unit/Genotyping Service of the Gulbenkian Institute of Science. A total of 40 SNPs were selected, of which 7 were excluded from analysis due to failure to design suitable primers. Thus, a plex of 33 SNPs was generated having in consideration the following genes: IL1α/β, IL-6, IL-10, IL-18, TGFβ1, IFNGRI, TNFα, DDX8, MAVS and FUT2.

Statistical analysis

All observed genotype frequencies of patients prior to transplantation were compared with expected genotype frequencies to test for deviations from Hardy-Weinberg equilibrium. Statistical analysis was performed with IBM SPSS Statistics for Mac, Version 24.0 (Armonk, NY: IBM Corp). The Chi-square ($\chi 2$) analysis was used to compare the categorical variables with a 5% significance level and Fisher Exact test (2-sided) was used when expected frequencies was < 5. The odds ratio (OR) and the corresponding 95% confidence intervals

(CIs) were estimated as a measure of association between the categorical variables. Logistic regression was performed in order to adjust the ORs to age and gender.

RESULTS

Population Characterization

This study was developed following two previous studies that evaluated the incidence of PTLD in our institution (Marinho-Dias, Baldaque et al. 2018, Marinho-Dias, Lobo et al. 2018). A total of 39 patients (15 females and 24 males) with different hematological malignancies were enrolled (Table 1C). The most frequent hematological malignancy were acute leukemias, accounting for 21 patients, and the majority underwent allogeneic hematopoietic stem cell transplant for the first time, with only one being enrolled for the second time.

Table 1C - Clinical-pathological data and transplant prophylaxis/regimen.

Characteristics	**N (%)**
Age, median (range) years old	27 (1-63)
Sex, n (%)	
Male	24 (61.5%)
Female	15 (38.5%)
Underlying disease, n (%)	
Aplastic anemia	3 (7.7%)
Acute leukemia	21 (53.8%)
Chronic leukemia	3 (7.7%)
Non-Hodgkin lymphoma	1 (2.6%)
Myelodysplastic/Myeloproliferative syndrome	6 (15.4%)
Others	5 (12.8%)
Conditioning regimen, n (%)	
Reduced Intensity	14 (35.9%)
Myeloablative	25 (64.1%)
ATG, n (%)	
Yes	22 (57.9%)
No	16 (42.1%)
Type of donor, n (%)	
Related	15 (38.5%)
Mismatched/Unrelated	24 (61.5%)
HLA identical	
Yes	33 (84.6%)
No	6 (15.4%)
Source of cells, n (%)	

Peripheral blood stem cells	32 (82.1%)
Bone marrow	3 (7.7%)
Umbilical cord blood	4 (10.3%)

EBV Infection and PTLD

Frequency of EBV infection was accessed in these patients, by monthly monitorization up to 6 months in peripheral blood samples. Analysis of EBV DNAemia (data not shown) revealed that 9 (23.1%) patients were first positive for EBV by the first month after transplant, 14 (35.9%) by 3 months and 6 (15.4%) by 6 months after transplant. Overall, 31 patients (79.5%) were positive for EBV at least once in the follow-up period.

In this group of patients, a total of 9 patients were diagnosed with PTLD. Nevertheless, the histological confirmation was only available for 2 cases. All other cases were based on clinical findings (e.g. fever, elevated LDH or hepatic enzymes and lymphadenopathies).

SNP Genotype Analysis

The evaluation of SNP genotypes was accessed by excluding SNPs that 1) did not provide results for >10% of the samples; and 2) samples with low quality DNA that were not suitable for genotyping, and therefore not presenting results for over 5% of SNP. Of the 33 SNPs only 22 SNPs matched these criteria and were used for analysis (Supplementary Table 1C). The Hardy-Weinberg equilibrium was accessed to identify SNPs with genotypic deviations from the expected in overall population. The analysis shows that with exception of rs1800469, rs2856838, rs3024505 and rs4937113, all other SNPs were in accordance with Hardy-Weinberg equilibrium ($p>0.05$) (Supplementary Table 2C). This test was performed in the genotypes detected in the samples collected prior transplantation.

The description of the different SNP genotypes in samples obtained pre- and post-transplant is shown in Table 2. We also evaluated the genotype change after transplant by comparing the individual genotypes pre and post-transplant (Table 2C). Results show that in the majority of SNPs there was >20% of cases that changed the genotype, particularly for rs281381 (30.6%), rs4937113 (31.4%), rs1878321 (32.4%) and rs2069727 (38.2%).

SNPs and EBV infection

We analyzed the association of post-transplant SNP genotypes and the development of EBV infection and results showed significant genotypic differences in genotype distribution of rs1143627 ($p=0.019$), rs1800629 ($p=0.061$) and rs2735097 ($p=0.087$) (Table 3C). The

statistical analysis revealed that rs1143627 A carrier genotypes are associated with a 14-fold increased risk of EBV infection (p=0.024; OR=14.0; 95%CI 1.63-120), which was confirmed by logistic regression adjusted for age and gender (p=0.016; OR=14.0; 95%CI 1.63-120) (data not shown).

We also analyzed the association of genotype change with EBV infection and no statistically significant result was found (Supplementary Table 3C).

SNPs and PTLD

Similarly, we analyzed the association of post-transplant SNP genotypes and the development of PTLD (Table 4C). Results showed significant differences in genotype distribution for rs2856838 (p=0.029), rs3024496 (p=0.056), rs6052130 (0.009) and rs281381 (0.098). The statistical analysis revealed increased risk for PTLD development associated with rs2856838 AA genotype (p=0.049; OR=1.28; 95%CI 0.91-1.82), rs3024496 GG genotype (p=0.037; OR=7.20; 95%CI 1.22-42.5) and rs6052130 A carriers (p=0.009; OR 1.50; 95%CI 0.95-2.38.) These data were not confirmed by logistic regression with adjustment for age and gender (data not shown).

The analysis of genotype change after transplantation and PTLD development revealed that genotype modification in the MAVS rs6052130 was associated with higher risk of PTLD (OR=1.50, *p*=0.010). Furthermore, results showed two SNPs (rs281381 and rs602662) with borderline significance showing that genotype change might be associated with a tendency for protection of PTLD development (Supplementary Table 4C).

Table 2C - SNP genotyping pre and post-transplant.

SNP (n_{pre}/n_{post})	Genotype	Pre-transplant n (%)	Post-transplant n (%)	Cases with genotype change, n (%)
rs1799964 (37/36)	CC	5 (13.5)	4 (11.1)	4 (11.8)
	CT	15 (40.5)	14 (38.9)	
	TT	17 (45.9)	18 (50.0)	
rs1143627 (38/36)	AA	16 (42.1)	18 (50.0)	9 (25.0)
	AG	18 (47.4)	13 (36.1)	
	GG	4 (10.5)	5 (13.9)	
rs1143633 (38/39)	CC	15 (39.5)	16 (41.0)	10 (26.3)
	CT	20 (52.6)	20 (51.3)	
	TT	3 (7.9)	3 (7.7)	
rs11436341 (38/38)	AA	2 (5.3)	3 (7.9)	10 (27.0)
	AG	12 (31.6)	13 (34.2)	
	GG	24 (63.2)	22 (57.9)	
rs1327474 (37/39)	CC	8 (21.6)	8 (20.5)	10 (27.0)
	CT	15 (40.5)	15 (38.5)	
	TT	14 (37.8)	16 (41.0)	
rs1800469 (38/39)	AA	4 (10.5)	2 (5.1)	9 (23.7)
	AG	10 (26.3)	17 (43.6)	
	GG	24 (63.2)	20 (51.3)	
rs1800629 (39/39)	AA	1 (2.6)	1 (2.6)	3 (7.7)
	AG	10 (25.6)	9 (23.1)	
	GG	28 (71.8)	29 (74.4)	
rs1878321 (35/38)	AA	17 (48.6)	15 (39.5)	11 (32.4)
	AG	15 (42.9)	18 (47.4)	
	GG	3 (8.6)	5 (13.2)	
rs2069727 (37/36)	CC	12 (32.4)	12 (33.3)	13 (38.2)
	CT	18 (48.6)	13 (36.1)	
	TT	7 (18.9)	11 (30.6)	
rs2069840 (38/38)	CC	12 (31.6)	16 (42.1)	9 (24.3)
	CG	21 (55.3)	19 (50.0)	
	GG	5 (13.2)	3 (7.9)	
rs2735097 (37/36)	AA	3 (8.1)	3 (8.3)	1 (2.9)
	AC	16 (43.2)	15 (41.7)	
	CC	18 (48.6)	18 (50.0)	
rs281381 (37/37)	CC	5 (13.5)	6 (16.2)	11 (30.6)
	CT	21 (56.8)	16 (43.2)	
	TT	11 (29.7)	15 (40.5)	
rs2856838 (39/39)	AA	2 (5.1)	2 (5.1)	10 (25.6)
	AG	20 (51.3)	23 (59.0)	
	GG	17 (43.6)	14 (35.9)	
rs3024496 (37/39)	AA	17 (45.9)	16 (41.0)	10 (27.0)
	AG	15 (40.5)	16 (41.0)	
	GG	5 (13.5)	7 (17.9)	
rs3024498 (37/37)	CC	---	1 (2.6)	7 (20.0)
	CT	9 (24.3)	7 (24.3)	
	TT	28 (75.7)	29 (75.7)	
rs3024505 (39/39)	AA	---	1 (2.6)	9 (23.1)
	AG	14 (35.9)	12 (30.8)	
	GG	25 (64.1)	26 (66.7)	
rs3783521 (38/37)	AA	5 (13.2)	4 (10.8)	7 (19.4)
	AG	18 (47.4)	12 (32.4)	
	GG	15 (39.5)	21 (56.8)	
rs4633144 (37/39)	CC	23 (62.2)	17 (43.6)	11 (29.7)
	CT	11 (29.7)	18 (46.2)	
	TT	3 (8.1)	4 (10.3)	
rs4937113 (36/38)	AA	15 (41.7)	9 (23.7)	11 (31.4)
	AT	11 (30.6)	14 (36.8)	
	TT	10 (27.8)	15 (39.5)	
rs602662 (38/39)	AA	9 (23.7)	11 (28.2)	11 (28.9)
	AG	18 (47.4)	16 (41.0)	
	GG	11 (28.9)	12 (30.8)	
rs6052130 (38/39)	CC	36 (94.7)	36 (92.3)	3 (7.9)
	CA	2 (5.3)	3 (7.7)	
	AA	---	---	
rs9376267 (36/37)	CC	22 (61.1)	23 (62.2)	7 (20.6)
	CT	11 (30.6)	8 (21.6)	
	TT	3 (8.3)	6 (16.2)	

Table 3C - SNP genotyping post-transplant and EBV infection.

SNP (n)	**Genotype**	**EBV negative n (%)**	**EBV positive n (%)**	**p**
rs1799964 (36)	CC	1 (14.3)	3 (10.3)	0.817
	CT	2 (28.6)	12 (41.4)	
	TT	4 (57.1)	14 (48.3)	
rs1143627 (36)	AA	2 (33.3)	16 (53.3)	0.019
	AG	1 (16.7)	12 (40.0)	
	GG	3 (50.0)	2 (6.7)	
rs1143633 (39)	CC	4 (50.0)	12 (38.7)	0.646
	CT	3 (37.5)	17 (54.8)	
	TT	1 (12.5)	2 (6.5)	
rs1143634 (38)	AA	----	3 (10.0)	0.458
	AG	2 (25.0)	11 (36.7)	
	GG	6 (75.0)	16 (53.3)	
rs1327474 (39)	CC	1 (12.5)	7 (22.6)	0.707
	CT	4 (50.0)	11 (35.5)	
	TT	3 (37.5)	13 (41.9)	
rs1800469 (39)	AA	1 (12.5)	1 (3.2)	0.197
	AG	5 (62.5)	12 (38.7)	
	GG	2 (25.0)	18 (58.1)	
rs1800629 (39)	AA	1 (12.5)	----	0.061
	AG	3 (37.5)	6 (29.4)	
	GG	4 (50.0)	25 (80.6)	
rs1878321 (38)	AA	2 (25.0)	13 (43.3)	0.442
	AG	4 (50.0)	14 (46.7)	
	GG	2 (25.0)	3(10.0)	
rs2069727 (36)	CC	3 (37.5)	9 (32.1)	0.922
	CT	3 (37.5)	13 (35.7)	
	TT	2 (25.0)	9 (32.1)	
rs2069840 (38)	CC	2 (28.6)	14 (45.2)	0.636
	CG	4 (57.1)	15 (48.4)	
	GG	1 (14.3)	2 (6.5)	
rs2735097 (36)	AA	----	3 (10.7)	0.087
	AC	6 (75.0)	9 (32.1)	
	CC	2 (25.0)	16 (57.1)	
rs281381 (37)	CC	1 (12.5)	5 (17.2)	0.897
	CT	4 (50.0)	12 (41.4)	
	TT	3 (37.5)	12 (41.4)	
rs2856838 (39)	AA	----	2 (6.5)	0.546
	AG	4 (50.0)	19 (61.3)	
	GG	4 (50.0)	10 (32.3)	
rs3024496 (39)	AA	3 (37.5)	13 (41.9)	0.821
	AG	4 (50.0)	12 (38.7)	
	GG	1 (12.5)	6 (19.4)	
rs3024498 (37)	CC	----	1 (3.4)	0.783
	CT	2 (25.0)	5 (17.2)	
	TT	6 (75.0)	23 (79.3)	
rs3024505 (39)	AA	----	1 (3.2)	0.389
	AG	4 (50.0)	8 (25.8)	
	GG	4 (50.0)	22 (71.0)	
rs3783521 (37)	AA	1 (12.5)	3 (10.3)	0.878
	AG	2 (25.0)	10 (34.5)	
	GG	5 (72.5)	16 (55.2)	
rs4633144 (39)	CC	3 (37.5)	14 (45.2)	0.920
	CT	4 (50.0)	14 (35.2)	
	TT	1 (12.5)	3 (9.7)	
rs4937113 (38)	AA	1 (12.5)	8 (26.7)	0.319
	AT	2 (25.0)	12 (40.0)	
	TT	5 (72.5)	10 (33.3)	
rs602662 (39)	AA	----	11 (35.5)	0.120
	AG	4 (50.0)	12 (38.7)	
	GG	4 (50.0)	8 (25.8)	
rs6052130 (39)	CC	8 (100)	28 (90.3)	1.000
	CA	----	3 (9.7)	
	AA	---	---	
rs9376267 (37)	CC	4 (57.1)	19 (63.3)	0.588
	CT	1 (14.3)	7 (23.3)	
	TT	2 (28.6)	4 (13.3)	

Table 4C - SNP genotyping post-transplant and PTLD infection.

SNP (n)	**Genotype**	**PTLD negative n (%)**	**PTLD positive n (%)**	**p**	**Risk model**	**p**	**OR (95%CI)**
	CC	4 (13.8)	----				
rs1799964 (36)	CT	11 (37.9)	3 (42.9)	0.580	Ccarrier vs TT	1.00	1.42 (0.27 – 7.58)
	TT	14 (48.3)	4 (57.1)				
	AA	13 (48.1)	5 (55.6)				
rs1143627 (36)	AG	10 (37.0)	2 (33.3)	0.920	Gcarrier vs AA	1.00	1.35 (0.30 – 6.13)
	GG	4 (14.8)	1 (11.1)				
	CC	12 (40.0)	4 (44.4)				
rs1143633 (39)	CT	16 (53.3)	4 (44.4)	0.853	CC vs Tcarrier	1.00	0.83 (0.18 – 3.75)
	TT	2 (6.7)	1 (11.1)				
	AA	2 (6.7)	1 (12.5)				
rs1143634 (38)	AG	10 (33.3)	3 (37.5)	0.813	GG vs Acarrier	0.698	1.50 (0.31 – 7.18)
	GG	18 (60.0)	4 (50.0)				
	CC	6 (20.0)	2 (22.2)				
rs1327474 (39)	CT	11 (36.7)	4 (44.4)	0.863	TT vs Ccarrier	0.711	1.58 (0.32 – 7.30)
	TT	13 (43.3)	3 (33.3)				
	AA	2 (6.7)	---				
rs1800469 (39)	AG	14 (46.7)	6 (66.6)	0.491	Acarrier vs GG	0.451	2.28 (0.48 – 10.9)
	GG	14 (46.7)	3 (33.3)				
	AA	1 (3.3)	----				
rs1800629 (39)	AG	8 (70.0)	1 (11.1)	0.507	Acarrier vs GG	0.400	3.42 (0.37 – 31.6)
	GG	21 (26.7)	8 (88.9)				
	AA	11 (37.9)	4 (44.4)				
rs1878321 (39)	AG	14 (48.3)	4 (44.4)	0.936	GG vs Acarrier	1.00	1.28 (0.12 – 13.2)
	GG	4 (13.8)	1(11.1)				
	CC	10 (37.0)	2 (22.2)				
rs2069727 (36)	CT	11 (40.7)	2 (22.2)	0.170	Ccarrier vs TT	0.096	4.38 (0.89 – 21.6)
	TT	6 (22.2)	5 (55.6)				
	CC	12 (41.4)	4 (44.4)				
rs2069840 (38)	CG	16 (55.2)	3 (33.3)	0.154	Ccarrier vs GG	0.134	8.00 (0.63 – 101)
	GG	1 (3.4)	2 (22.2)				
	AA	2 (6.9)	1 (14.3)				
rs2735097 (36)	AC	12 (41.4)	3 (42.9)	0.794	Ccarrier vs AA	0.488	2.25 (0.42 – 14.2)
	CC	15 (51.7)	3 (42.9)				
	CC	6 (20.7)	----				
rs281381 (37)	CT	10 (34.5)	6 (75.0)	0.098	TT vs Ccarrier	0.431	2.44 (0.42 – 14.2)
	TT	13 (44.8)	2 (25.0)				
	AA	----	2 (22.2)				
rs2856838 (39)	AG	19 (63.3)	4 (44.4)	0.029	Gcarrier vs AA	0.049	1.28 (0.91 – 1.82)
	GG	11 (36.7)	3 (33.3)				
	AA	13 (43.3)	3 (33.3)				
rs3024496 (39)	AG	14 (46.7)	2 (22.2)	0.056	Acarrier vs GG	0.037	7.20 (1.22 – 42.5)
	GG	3 (10.0)	4 (44.4)				
	CC	----	1 (12.5)				
rs3024498 (37)	CT	6 (20.7)	1 (12.5)	0.145	TT vs Ccarrier	1.00	1.28 (0.20 – 8.00)
	TT	23 (79.3)	6 (75.0)				
	AA	1 (3.3)	----				
rs3024505 (39)	AG	9 (30.0)	3 (33.3)	0.850	Acarrier vs GG	1.00	1.00 (0.21 – 4.86)
	GG	20 (66.7)	6 (66.7)				
	AA	4 (13.3)	----				
rs3783521 (37)	AG	10 (33.3)	2 (28.6)	0.522	A carrier vs GG	0.674	2.19 (0.36 – 13.2)
	GG	16 (53.3)	5 (71.4)				
	CC	14 (46.7)	3 (33.3)				
rs4633144 (39)	CT	13 (43.3)	5 (55.6)	0.733	CC vs Tcarrier	0.704	1.75 (0.36 – 8.39)
	TT	3 (10.0)	1 (11.1)				
	AA	6 (20.0)	3 (37.5)				
rs4937113 (38)	AT	13 (43.3)	1 (12.5)	0.255	Tcarrier vs AA	0.363	2.40 (0.44 – 13.0)
	TT	11 (36.7)	4 (50.0)				
	AA	8 (26.7)	3 (33.3)				
rs602662 (39)	AG	11 (36.7)	5 (55.6)	0.336	GG vs Acarrier	0.228	4.63 (0.51 – 42.1)
	GG	11 (36.7)	1 (11.1)				
	CC	30 (100)	6 (66.7)				
rs6052130 (39)	CA	----	3 (33.3)	0.009	CC vs Acarrier	0.009	1.50 (0.95 – 2.38)
	AA	---	---				
	CC	15 (53.6)	8 (88.9)				
rs9376267 (37)	CT	7 (25.0)	1 (11.1)	0.142	Tcarrier vs CC	0.112	6.90 (0.76 – 63.0)
	TT	6 (21.6)	----				

DISCUSSION

EBV infection, in the period subsequent to allogeneic stem cell transplantation, is frequent and is responsible for uncontrolled B cell proliferation in an environment with diminished or absence of immunologic surveillance (Janani, Malathi et al. 2015, Fan, Jing et al. 2016, Marinho-Dias, Baldaque et al. 2018). PTLDs are rare events and rather deadly in these patients, which makes it important to identify correctly patients at risk (Marinho-Dias, Lobo et al. 2018). In patients submitted to allo-HSCT, there have been identified several risk factors, such as HLA-mismatched, unrelated donor, mismatched seropositivity (D+/R-), active EBV infection, use of anti-thymocyte globulin and use of myeloablative regimens (Marinho-Dias, Lobo et al. 2018). These clinical data are not enough to determine which patients are at higher risk, and therefore it is important to identify potential biomarkers that could help in the prediction of EBV infection or PTLD development.

In our study, we verified that EBV infection was significantly frequent in our population, since 79.5% of all patients were positive at least once in the period post-transplant, with a peak at 2 months (76.9%). This is consistent with our previous study and other reports, where EBV DNAemia reached 70.0%, confirming that we have a significant rate of infection in our allo-HSCT recipients (Schonberger, Meisel et al. 2010, Liu, Xuan et al. 2013, Marinho-Dias, Baldaque et al. 2018).

The main aim of this study was to evaluate the role of genetic polymorphism in the definition of a susceptibility profile to EBV infection and consequently PTLD development in allo-HSCT. We have developed a strategy to identify potential SNPs that represent haplotypes, and which could represent a significative part of host genome immune response. In the study we included a total of 33 SNPs from HLA polymorphic sites (class I, II and III) and non-HLA regions including host immune response genes (such as TNFA, IL-1a, IL-1b, IL-1RN, IL10, IFNG, IFNGRI and IFNGRII); antiviral effectors (such as RIG-I, DDX58, MDA-5, IFIH1, MAVS, VISA, IPS1, CARDIF and ISG15 ubiquitin-like pathway); and cellular glycoprotein (FUT2/3). After the study we were only able to retrieve reliable data from 22 SNPS in different genes associated with immune response (IL1α/β, IL-6, IL-10, IL-18, TGFβ1, IFNGRI, TNFα, DDX8, MAVS and FUT2). Since all these SNPs were selected with a tight linkage disequilibrium, it is important to take into account that each SNP represents a locus of several SNPs.

Several SNPs, associated with EBV infection and PTLD development, have been studied, but mainly regarding solid transplantation. Namely IL-28B (rs12979860), where the CC genotype might influence the anti-viral response from IFNG (Akay, Patel et al. 2014); HLA-A26, B38 haplotype, both in the donor and recipient, predisposes the patient to PTLD

development in the subset of solid organ transplant (Reshef, Luskin et al. 2011); and TNF promoter -1031C and -863A variant alleles have been linked to a higher risk of PTLD development in heart transplant patients (McAulay, Haque et al. 2009, Morscio, Dierickx et al. 2013). Furthermore, HLA-A2, A11, B5 and B35, concomitant with azathioprine, are associated with higher risk of PTLD and (Pourfarziani, Einollahi et al. 2007). Finally, when studying PTLD development in mice, IFNG +874 T>A polymorphism, the AA genotype was linked to early onset EBV-driven PTLD (Dierksheide, Baiocchi et al. 2005).

One of the important things in this study is that we have analyzed the genotypes pre- and post-transplant. This is particularly important since, despite HLA-match, after transplant the recipient will have an immune system based on the donor, and therefore the genetic susceptibility should be tested at the time of engraftment confirmation. Our data analysis revealed that variation in genotype after transplantation was observed in the majority of SNPs particularly for FUT 2 rs281381 (30.6%), IL-18 rs4937113 (31.4%), IL-1A rs1878321 (32.4%) and IFNG rs2069727 (38.2%). FUT2, also known as fucosyltransferase 2 gene, is responsible for encoding Lewis antigens related to the ABO blood group. FUTs are involved in many biological processes such as cell adhesion and tumor progression (Chen, Liao et al. 2017). FUT2 has been recently associated with an increased risk of GVHD after HSCT by genetically modifying the gut microbiome (Rayes, Morrow et al. 2016). IL-18, also designated as interleukin-18 gene, encodes a proinflammatory cytokine that enhances natural killer cell activity in spleen cells and stimulates interferon gamma production in T-helper type I cell (Carroll, Paunovic et al. 2008). IL-18 is known to have synergistic effect, along with IL-12, which reduces T-cell mediated GVHD in mice (Leung, Iyengar et al. 2004). IL-1A has been known to play a major role in immune response, inflammatory processes and hematopoiesis, and its deregulated signaling leads to severe acute or chronic inflammation, such as GVHD (Park, Lee et al. 2015, Di Paolo and Shayakhmetov 2016, de Mooij, Netea et al. 2017). IFNG is a proinflammatory cytokine and plays an important role in innate and adaptive immunity against infections and tumor development, by modulating the function of hematopoietic stem cells, mainly by inhibiting their production (de Bruin, Voermans et al. 2014). IFNG has been used as a therapy for PTLD, where IFNG secreting T-cells of the donor are isolated and infused in the patient, and it has shown to be effective in early stages of PTLD (Bollard and Heslop 2016). IFNG has also been associated with EBV-driven tumors, where viral lytic proteins favor immune evasion by inhibiting IFNG production and therefore contribute to tumorigenesis (Petrara, Giunco et al. 2015). Despite these evidences, when evaluating genotypic changes associated with EBV infection, no statistically significant results were attained; while for PTLD development we have only verified an association of genotype change in MAVS rs6052130 (OR=1.50, p=0.010).

Additionally, FUT2 rs281381 and rs602662, presented borderline statistically significant results for a protective effect of PTLD development after genotype alterations. These results support the need for further studies to characterize the role of these proteins in EBV infection and PTLD development and to evaluate its utility in allo-HSCT patients management.

The independent analysis of SNPs revealed that IL-1B rs1143627 Acarriers have a significant higher risk of EBV infection (OR=14.0, *p*=0.024). IL-1B polymorphisms have been strongly associated with the development of cancer, namely lung and prostate cancer, because of its pro-inflammatory effect (Andersen, Holst et al. 2013). In lung cancer, a study from Zienolddiny *et al.*, revealed an association between Acarrier subjects and a mutation in the *p53* gene, with the development of lung cancer and the risk was higher when homozygosity was present (AA genotype) (Zienolddiny, Ryberg et al. 2004). As for the association with viral infections, literature shows that a relation Acarriers have increased susceptibility to pandemic H1N1 influenza A virus (Liu, Li et al. 2013). Furthermore, there are other studies that show that same SNP is associated with hepatitis B vaccine immune response (heterozygotes associated with non-response to vaccination) and Chronic Hepatitis B virus infection (GG genotype) (Chen, Liang et al. 2011, Wu, Song et al. 2018). This is the first study to show an association of this SNP with EBV infection and results show a clear association that should be further confirmed in larger series since the predictive risk (14-fold increase) may be of significant importance for clinical management and the genotype of donors regarding the SNP may be useful to select the best follow-up and prophylaxis for allo-HSCT recipients.

Regarding the development of PTLD we found that 3 SNPs are associated with increased risk: IL-1A rs2856838 AA genotype (OR=1.28, *p*=0.049); IL-10 rs3024496 GG genotype (OR=7.20, *p*=0.037): and MAVS rs6052130 A carriers (OR=1.50, *p*=0.009). IL-1A rs2856838 has not been studied extensively, although one study reports an association between the AA genotype and a protective effect on chronic rhinosinusitis (Mfuna Endam, Cormier et al. 2010); other reveals association of heterozygotic genotype and increased susceptibility for tuberculosis (Wang, Jin et al. 2018); and another associates heterozygotic and AA genotype with lower risk of contracting malaria (Legason, Pfeiffer et al. 2017). As for IL-10 rs3024496 GG genotype has been linked with prostate and colorectal cancer (Tsilidis, Helzlsouer et al. 2009, Wang, Helzlsouer et al. 2009), but also to HCMV infection (Loeffler, Steffens et al. 2006). Nevertheless, there are controversial studies regarding this SNP and the development of tuberculosis (Shin, Park et al. 2005, Moller, Nebel et al. 2010). Finally, MAVS rs6052130 CA genotype has been reported as a risk factor for development

of cervical precancerous lesions as well as increased vulnerability to high-risk HPV-induced cervical precancerous lesions (Xiao, Liu et al. 2018).

To the best of our knowledge, this is the first study concerning genotype alterations of these SNPs and its association with EBV infection and PTLD development in allo-HSCT recipients. Despite having a small population, the study clearly identifies IL-1B rs1143627 (Acarriers) as a significant risk factor for EBV infection and IL-1A rs2856838 (AA genotype), IL-10 rs3024496 (GG genotype) and MAVS rs6052130 (Acarriers) as risk markers for PTLD development. To clarify these data, more studies should be performed in larger series and testing other SNPs from same genes.

SUPPLEMENTARY TABLES

Supplementary Table 1C - Description of single nucleotide polymorphisms (SNP) selected for the study.

SNP	Alleles	Gene	Chr location	Chr. position	Alternative ID	Region	Reference allele frequency
rs1143627	G/A	IL-1B	2q13	112836810	-31 G>A	UTR-5	G - 0.36; A - 0.64
rs1143633	T/C	IL-1B	2q13	112832890	8890 T>C	Intron	T - 0.40; C - 0.60
rs1143634	G/A	IL-1B	2q13	112832813	8967 C/T	Coding	G - 0.79; A - 0.21
rs2069840	C/G	IL-6	7p15.3	22768572	6807 C>G	Intron	G - 0.29; C - 0.71
rs3024496	A/G	IL-10	1q32	206768519	8976 T>C	UTR-3	G - 0.52; A - 0.48
rs3024498	T/C	IL-10	1q32	206941529	9311 A>G	UTR-3	T - 0.72; C - 0.28
rs3024505	T/C	IL-10	1q32.1	206766559	10936 C>T	MHC locus	C - 0.82; T - 0.18
rs4937113	A/T	IL-18	11q23.1	112158998	10120 T>A	Intron	T - 0.62; A - 0.38
rs1878321	A/G	IL-1A	2q13	112786857	-2423 T>C	5' upstream	C - 0.31; T - 0.69
rs2856838	G/A	IL-1A	2q13	113539972	8000 C>T	Intron	G - 0.60; A - 0.40
rs3783521	A/G	IL-1A	2q13	112786000	4395 C>T	5' upstream	G - 0.67; A - 0.33
rs2735097	C/A	HLA-A	6p21	29947524	10060 C>A	MHC locus	G - 0.61; T - 0.39
rs2069727	A/G	INFG	12q15	68154443	10299 A>G	3' downstream	A - 0.57; G - 0.43
rs9376267	C/T	INFGRI	6q23.2	137209894	14537 G>A	Intron	C - 0.71; T - 0.29
rs1327474	C/T	IFNGRI	6q23.3	137219938	-4493 G>A	UTR-5	T - 0.60; C - 0.40
rs1800469	A/G	TGFB1	19q13.2	41354391	4536 C>T	5' upstream	C - 0.71; T - 0.29
rs1800629	G/A	TNF	6p21.3	31575254	4682 G>A	5' upstream	G - 0.83; A - 0.17
rs1799964	C/T	TNF	6p21.3	31574531	3959 T>C	5' upstream	C - 0.21; T - 0.79
rs4633144	T/C	DDX58	9p21	325042296	27029 A>G	Intron	C - 0.64; T - 0.36
rs281381	C/T	FUT2	19q13.3	48711491	48711491 T>C	Intron	C - 0.36; T - 0.64
rs602662	G/A	FUT2	19q13.3	48703728	Gly258Ser	Exon	A - 0.61; G - 0.39
rs6052130	C/A	MAVS	20p23	3863021	21223 C>A	Intron	C - 0.97; A - 0.03

SNP, Single Nucleotide Polymorphism; Chr, Chromosome; UTR, Untranslated region; G, guanine; C, cytosine; T, thymidine; A, adenine.

Supplementary Table 2C **–** Hardy-Weinberg Equilibrium.

SNP	Pre-transplant genotypes	Hardy-Weinberg
rs1799964	CC (13.5%), TC (40.5%), TT (45.9%)	*p*=0.348
rs1143627	AA (42.1%), AG, 18 (47.4%), GG, 4 (10.5%)	*p*=0.594
rs1143633	CC, 15 (39.5%), TC, 20 (52.6%), TT, 3 (7.9%)	*p*=0.092
rs1143634	AA, 2 (5.3%), AG, 12 (31.6%), GG, 24 (63.2%)	*p*=0.608
rs1327474	CC, 8 (21.6%), CT, 15 (40.5%), TT, 14 (37.8%)	*p*=0.094
rs1800469	AA, 4 (10.5%), GA, 10 (26.3%), GG, 24 (63.2%)	***p*=0.006**
rs1800629	AA, 1 (2.6%), GA, 10 (25.6%), GG, 28 (71.8%)	*p*=0.861
rs1878321	AA, 17 (48.6%), AG, 15 (42.9%), GG, 3 (8.6%)	*p*=0.841
rs2069727	CC, 12 (32.4%), TC, 18 (48.6%), TT, 7 (18.9%)	*p*=0.929
rs2069840	CC, 12 (31.6%), GC, 21 (55.3%), GG, 5 (13.2%)	*p*=0.151
rs2735097	AA, 3 (8.1%), CA, 16 (43.2%), CC, 18 (48.6%)	*p*=0.727
rs281381	CC, 5 (13.5%), CT, 21 (56.8%), TT, 11 (29.7%)	*p*=0.096
rs2856838	AA, 2 (5.1%), AG, 20 (51.3%), GG, 17 (43.6%)	***p*=0.041**
rs3024496	AA, 17 (45.9%), GA, 15 (40.5%), GG, 5 (13.5%)	*p*=0.348
rs3024498	CC ---, CT, 9 (24.3%), TT, 28 (75.7%)	*p*=0.166
rs3024505	AA ---, GA, 14 (35.9%), GG, 25 (64.1%)	***p*=0.028**
rs3783521	AA, 5 (13.2%), AG, 18 (47.4%), GG, 15 (39.5%)	*p*=0.863
rs4633144	CC, 23 (62.2%), CT, 11 (29.7%), TT, 3 (8.1%)	*p*=0.109
rs4937113	AA, 15 (41.7%), TA, 11 (30.6%), TT, 10 (27.8%)	***p*<0.001**
rs602662	AA, 9 (23.7%), AG, 18 (47.4%), GG, 11 (28.9%)	*p*=0.621
rs6052130	CC, 36 (94.7%), CA, 2 (5.3%), AA ---	*p*=0.785
rs9376267a	CC, 22 (61.1%), CT, 11 (30.6%), TT, 3 (8.3%)	*p*=0.130

p, pearson coefficient by Chi-square analysis; G, guanine; C, cytosine; T, thymidine; A, adenine.

Supplementary Table 3C - SNP genotype change between pre and post-transplant and EBV infection.

SNP	Genotype change		p	OR, 95% CI
	EBV negative n/total (%)	EBV positive n/total (%)		
rs1799964	0/7 (0.0)	4/27 (14.8)	0.559	1.17 (1.00 – 1.37)
rs1143627	2/6 (33.3)	7/30 (23.3)	0.627	0.61 (0.09 – 4.06)
rs1143633	2/8 (25.0)	8/30 (26.7)	1.00	1.09 (0.18 – 6.56)
rs1143634	1/7 (14.3)	9/30 (30.0)	0.647	2.57 (0.27 – 24.5)
rs1327474	3/8 (37.5)	7/29 (24.1)	0.655	0.53 (0.10 – 2.80)
rs1800469	3/7 (42.9)	6/31 (19.4)	0.322	0.32 (0.06 – 1.83)
rs1800629	0/8 (0.0)	3/31 (9.7)	1.00	1.11 (0.99 – 1.24)
rs1878321	3/5 (60.0)	8/29 (27.6)	0.300	0.25 (0.04– 1.81)
rs2069727	4/8 (50.0)	9/26 (34.6)	0.679	0.53 (0.11 – 2.63)
rs2069840	0/7 (0.0)	9/30 (30.0)	0.160	1.42 (1.13 – 1.81)
rs2735097	0/8 (0.0)	1/27 (3.7)	01.00	1.04 (0.96 – 1.12)
rs281381	4/8 (50.0)	7/28 (25.0)	0.214	0.33 (0.06 – 1.70)
rs2856838	2/8 (25.0)	8/31 (25.8)	1.00	1.04 (0.17 – 6.26)
rs3024496	4/8 (50.0)	6/29 (20.7)	0.174	0.26 (0.05 – 1.36)
rs3024498	2/8 (25.0)	5/27 (18.5)	0.648	0.68 (0.10 – 4.43)
rs3024505	2/8 (25.0)	7/31 (22.6)	1.00	0.87 (0.14 – 5.34)
rs3783521	2/8 (25.0)	5/28 (17.9)	0.639	0.65 (0.10 – 4.23)
rs4633144	2/6 (33.3)	9/31 (29.0)	1.00	0.82 (0.13 – 5.29)
rs4937113	4/7 (57.1)	7/28 (25.0)	0.171	0.25 (0.04 – 1.40)
rs602662	3/8 (37.5)	8/30 (26.7)	0.667	0.61 (0.12 – 3.14)
rs6052130	0/8 (0.0)	3/30 (10.0)	1.00	1.11 (0.99 – 1.25)
rs9376267	2/6 (33.3)	5/28 (17.9)	0.580	0.44 (0.62 – 3.07)

Supplementary Table 4C - SNP genotype change between pre and post-transplant and PTLD

SNP	Genotype change		p	OR (95% CI)
	PTLD negative n/total (%)	PTLD positive n/total (%)		
rs1799964	4/28 (14,3)	0/6 (0.0)	1.00	0.86 (0.74 – 0.99)
rs1143627	6/27 (22.2)	3/9 (33.3)	0.660	1.75 (0.33 – 9.17)
rs1143633	6/30 (20.0)	4/8 (50.0)	0.170	4.00 (0.77 – 20.8)
rs1143634	6/29 (20.7)	4/8 (50.0)	0.174	3.83 (0.74 – 19.9)
rs1327474	8/28 (28.6)	2/9 (22.2)	1.00	0.71 (0.12 – 4.20)
rs1800469	8/29 (27.6)	1/9 (11.1)	0.411	0.33 (0.04 – 3.06)
rs1800629	1/30 (3.3)	2/9 (22.2)	0.127	8.29 (0.66 – 104.9)
rs1878321	8/26 (30.8)	3/8 (37.5)	1.00	1.35 (0.26 – 7.07)
rs2069727	9/25 (36.0)	4/9 (44.4)	0.704	1.42 (0.30 – 6.69)
rs2069840	6/29 (20.7)	3/8 (37.5)	0.373	2.30 (0.42 – 12.4)
rs2735097	1/28 (3.6)	0/7 (0.0)	1.00	0.96 (0.90 - 1.04)
rs281381	11/29 (37.9)	0/7 (0.0)	0.076	0.62 (0.47 – 0.83)
rs2856838	6/30 (20.0)	4/9 (44.4)	0.197	3.20 (0.65 – 15.7)
rs3024496	8/29 (27.6)	2/8 (25.0)	1.00	0.88 (0.15 – 5.27)
rs3024498	4/28 (14.3)	3/7 (42.9)	0.125	4.50 (0.72 – 28.1)
rs3024505	7/30 (23.3)	2/9 (22.2)	1.00	0.94 (0.16 – 5.59)
rs3783521	6/29 (20.7)	1/7 (14.3)	1.00	0.64 (0.06– 6.37)
rs4633144	8/28 (28.6)	3/9 (33.3)	1.00	1.25 (0.25 – 6.26)
rs4937113	9/27 (33.3)	2/8 (25.0)	1.00	0.67 (0.11 – 3.99)
rs602662	11/30 (36.7)	0/8 (0.0)	0.077	0.63 (0.48 – 0.83)
rs6052130	0/29 (0.0)	3/9 (3.4)	0.010	1.50 (0.95 – 2.38)
rs9376267	5/25 (20.0)	2/9 (22.2)	1.00	1.14 (0.18 – 7.28)

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GENERAL DISCUSSION

Leukemia and Lymphomas are amongst the cancer types that have been increasing in the past decades (Howlader, Noone et al. 2017) The use of hematopoietic stem cell transplantation as an approach to the treatment for hematological malignancies represents one of the greatest advances in medicine. Nevertheless, these patients are exposed to significant morbidity and mortality due to the critical immunocompromised status during the pre- and post-transplant period, especially those submitted to allogeneic HSCT (Safdar 2011).

Viral infections are one of the most common complications during the post-transplant period and the identification of groups of individuals with increased risk of developing a viral infection is crucial (Lin and Liu 2013, Srinivasan, Wang et al. 2013). EBV is one of the most common viral infections during this period and is associated with the period of major immunosuppression, which is from 1 month to 1 year (Jha 2010). The monitoring of EBV status during pre- and post-transplant period is still not performed in all cases, probably due to the low number of severe EBV-associated complications (Allen, Alfieri et al. 2002, Gulley and Tang 2010). Indeed, EBV infection is associated with the development of PTLD in HSCT patients, and this represents the major problem of EBV infection (Styczynski, van der Velden et al. 2016).

This thesis intends to clarify EBV infection in HSCT patients and to evaluate associated risk factors and the ability to predict the risk of EBV and PTLD development based on genetic markers. The study was performed in three distinct parts including 1) a retrospective study to characterize PTLD cases; 2) a prospective study to characterize EBV infection and its association with clinicopathological data of patients; and 3) a prospective study to evaluate the genetic susceptibility for EBV infection based on the genetic profile of HSCT patients.

PTLD and HSCT

The retrospective study of PTLD in aHSCT at our institution allowed us to identify a total of 15 patients that developed PTLD between 2008 and 2012. The analysis revealed that PTLD development was not associated with age at transplantation neither with the myeloablative or reduced intensity regimens. On the other hand, we observed that PTLD was more frequent in patients who received grafts from unrelated donors. As described by Landgreen et al., unrelated donors leads to a 4-fold increase in risk of PTLD development (Landgren, Gilbert et al. 2009) Uhlin et al., in a retrospective study of risk factor for PTLD development, verified than in 40 patients who developed PTLD, 22 (55.0%) were from aHSCT from

unrelated donors, although with no statistical significance (Uhlin, Wikell et al. 2014). PTLD is often associated with increased risk of mortality (Bakker, van Imhoff et al. 2007). In our series the mortality rate was of 46.7%, which is consistent by previous studies (Preiksaitis 2004, Park, Choi et al. 2006), although other studies demonstrate higher rates (Curtis, Travis et al. 1999, Al-Mansour, Nelson et al. 2013). We observed that the most significant factor associated with mortality was the use of unrelated donors.

EBV monitoring in HSCT

EBV infection is, most of the times, directly correlated with PTLD and therefore the detection of EBV is predictive of PTLD occurrence (San-Juan, Comoli et al. 2014). The retrospective study with PTLD patients revealed that, the mean time for the detection of viral load was 68 days (mean 130 days; range 29-464 days), and 60.0% of these patients had infection on the early-period post-transplant, while the remaining had infection after 90 days. This data, when comparing to a study from Fan et al., reveals that our infection period is later than what is described (mean 45 days; range 14-88 days) (Fan, Jing et al. 2016). Janani et al showed that PTLD was diagnosed approximately in the same period of EBV infection. (Janani, Malathi et al. 2015). EBV viral loads at first detection were 4.9 $\log_{10}$ copies/mL (median 4.7 $\log_{10}$ copies/mL; range 3.3-7.0 $\log_{10}$ copies/mL), which is not very different from what has been reported by Janani et al., observed that PTLD patients had at the time of diagnosis EBV viral loads of 5.20 $\log_{10}$ copies/mL (Janani, Malathi et al. 2015). Gärtner at al., when studying EBV viral load to monitor PTLD development, observed that EBV positive patients without PTLD revealed viral loads of 3.6 $\log_{10}$ copies/mL (range 2.5-6.8 $\log_{10}$ copies/mL) and patients with PTLD had mean viral loads of 6.1 $\log_{10}$ copies/mL (range 3.1-8.0 $\log_{10}$ copies/mL) (Gartner, Fischinger et al. 2002).

The prospective study was developed to analyze the impact of EBV monitoring in HSCT management. Considering that viral infections usually start arising at 30 days posttransplant (Safdar 2011), we collected samples at day 30, 60, 90, 120, 150 and 180 posttransplant. This is a critical period since immune reconstitution is not yet established and T cell surveillance is impaired (Janeczko, Mielcarek et al. 2016). In our study, we had 38.3% samples positive for EBV, and the period with higher frequency of infection was between day +60 and +90. Our findings are consistent with data previously described, indicating EBV reactivation is more frequent between day 60 and 90 post-transplant (Ocheni, Kroeger et al. 2008). EBV infection was more frequently found in women and in adult population.

As PTLD is nearly always EBV-related, viral load monitoring can be applied as predictive of PTLD development (Gulley and Tang 2008, Gulley and Tang 2010). The median onset of

PTLD is 2 months, which is consistent with the occurrence of viral infections (Gulley and Tang 2010, Safdar 2011). Studies suggest that EBV viral load must be monitored weekly, starting at day +15 of transplant until day +100 (Coppoletta, Tedone et al. 2011, Styczynski, van der Velden et al. 2016). Other authors, recommend weekly monitoring until day +90 and then monthly until 1 year after transplantation and define a cut-off value of over 4 $\log_{10}$ copies/mL, in two subsequent samples, as a predictive factor for PTLD development (Comoli, Basso et al. 2007, Uhlin, Wikell et al. 2014). Indeed there is no consensus on which are the corrected cut-off values for predictive purposes (Faraci, Caviglia et al. 2010).

EBV infection and risk factors in allo-HSCT

In the prospective study we have analyzed EBV infection and the clinic-pathological characteristics of patients involved in the study. We observed that patients with unrelated and/or mismatched donors were more prone to develop an EBV infection, and similar findings were found for the use of peripheral blood as source of stem cells. The intensity of conditioning regimen has been shown to directly affect the relapse and survival of patients who undergo aHSCT (Atilla, Atilla et al. 2017). In our series, we found that myeloablative conditioning was associated with a 3-fold increased risk of developing EBV infection. These findings are according with those described by Xuan et al. that observed that intensified conditioning might be increase the incidence of EBV viremia and disease. Similar results were found for the use of ATG in the conditioning regimen (Xuan, Huang et al. 2012).

When addressing the development of GVHD, we observed that patients who developed aGVHD, were 3 times more prone for EBV infection. Furthermore, we noticed that all patients with cGVHD, were positive for EBV at least once in the post-transplant follow-up. As described previously by Janeczko et al., GVHD is related to delayed immune reconstitution, favoring infections in the early period post-transplant. Moreover, viral infections are also associated with delayed immune reconstitution and appear to be linked to the degree of immunosuppression. Therefore, monitoring of infections is critical in this phase (Janeczko, Mielcarek et al. 2016).

Other interesting data from this prospective study was that we found that some risk factors were differentially associated with EBV infection over time. At 60 days post-transplant, we verified that EBV infection was associated with transplants from unrelated donors OR=3.9, p=0.058), myeloablative conditioning (OR=4.3, p=0.052), and ATG use (OR=3.6, p=0.099). At 90 days only GVHD was related a higher risk of infection (OR=6.7, p=0.032). Finally, at 150 days, risk of infection was related with unrelated donors (OR=8.0, p=0.043), ATG (OR=12.0, p=0.03) and GVHD (OR=5.6, p=0.099). Indeed, the cox regression analysis

considering gender, type of donor, conditioning regimen, use of ATG and development of aGVHD, we observed that there is an association of unrelated donor and EBV infection at 150 days post-transplant (HR=8.8, *p*=0.03).

In this series, despite the existence high rates of EBV infection, none of the patients included in the prospective study developed PTLD. One possible explanation for this is the use of Rituximab, either before transplant, as a prophylactic and preemptive approach, and when there is evidence of EBV infection. Rituximab is a monoclonal antibody targeted to the protein CD20, which is present on the surface of B lymphocytes, therefore inhibits EBV proliferation. Indeed, there are several authors that have been showing the utility of Rituximab to control PTLD. Wakabayashi et al. reported an EBV-associated PTLD following HSCT from an unrelated donor, with EBV DNAemia at 30 days after transplant. Weekly monitoring of EBV viral load was not effective in preventing PTLD. Administration of rituximab was effective in PTLD regression (Wakabayashi, Ohashi et al. 2010).. Therefore, as previous studies suggested, Rituximab can be used safety and with efficacy in the treatment for EBV-related PTLD. Another explanation for this absence of PTLD in our series, is the use of rabbit ATG instead of equine ATG, which was administered to patients from the retrospective study. According to Storek et al., rabbit ATG is responsible for profound and long-lasting lymphocytopenia. When comparing to the equine, rabbit ATG was effective in GVHD prophylaxis, while the equine version was not. Moreover, when analyzing a randomized study from Champlin et al., incidence of cGVHD was higher when using equine ATG when compared to patients without ATG. Storek et al., shows evidence that rabbit ATG, at low doses, promotes overall survival, and decreases rates of GVHD when patients are submitted to myeloablation to BM or PBSC transplant, suggesting rabbit ATG might be used as GVHD prophylaxis. These findings are supported by Yuan et al., in a meta-analysis of the actions of rabbit vs. equine ATG (Champlin, Perez et al. 2007, Storek, Mohty et al. 2015, Yuan, Pei et al. 2017).

Finally, it is important to state that our mortality rates were 46.7% and 40.0%, in the retrospective and prospective study, respectively. These are consistent with a study from Styczynski et al., that have reported, in a much larger population, a death rate of 45.2% (in a follow-up period of 4.9 years) (Styczynski, Tridello et al. 2016).

SNPs and EBV infection and PTLD development

The analysis of SNPs as genetic risk markers of different diseases/infections has been widely studied, and these studies focus in identifying biomarkers that can help in prevention, diagnosis and treatment of several diseases (Gu et al. 2018, Erichsen et al. 2004). In our

study we aimed to identify potential biomarkers for predicting EBV infection/reactivation and PTLD development.

Association between genetic polymorphisms and immune response have been widely studied. Therefore, we hypothesized an association between certain polymorphisms of the immune response and EBV infection/reactivation as well as PTLD development. With this we developed aa 33 SNP microarray plex, while studying patients genotypes pre- and post-transplantation. We concluded that there is an association of higher risk between IL-1B rs1143627 Acarriers and the development of EBV infection post-transplantation. When reviewing the results regarding PTLD development, three specific genotypes were associated with higher risk of PTLD development: IL-1A rs2856838 AA genotype, IL-10 rs3024496 GG genotype and MAVS rs6052130 Acarriers. This information leads us to hypothesize that there might be more SNPs associated with this matter. Since our population is small, further studies with larger populations and multicentric approaches are required to complement/emphasize these findings.

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CONCLUSIONS

As conclusions of our study, I would like to emphasize the importance in EBV monitoring in patients submitted to transplant. EBV infection, when associated with other transplant-related complications, may lead to poor outcomes with high rates of morbidity and mortality. Patients with risk factors such as unrelated donor, mismatched donor, EBV seronegative prior to transplant, development of GVHD, use of ATG, and subsequent PTLD, should be carefully monitored for viral infections. Furthermore, polymorphism analysis is important in the subset of transplantation, mainly by identifying patients with higher risk of EBV infection and PTLD development. IL-1B rs1143627 (Acarriers) increases EBV infection risk and IL-1A rs2856838 (AA genotype), IL-10 rs3024496 (GG genotype) and MAVS rs6052130 (Acarriers) are risk markers for PTLD development and should be further studied in larger populations. PTLD remains a major issue in the subset of transplant, and fortunately, at our institution, progresses in this matter have been made since none of our prospective study patients developed PTLD.

ATTACHMENT I – Journal Article (Study I)

Post-transplant lymphoproliferative disorder in hematopoietic stem cell transplant patients: A single center retrospective study between 2005 and 2012

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Abstract. Post-transplant lymphoproliferative disorder (PTLD), despite its rarity, is an important mortality/morbidity event in transplant patients. The purpose of the present study was to retrospectively examine the clinical and pathologic characteristics, and outcomes of PTLD at the Portuguese Oncology Institute of Porto. A retrospective review of patient information was performed for patients that developed PTLD following allogeneic hematopoietic stem cell transplant (aHSCT) and were diagnosed between 2005 and 2012. The present study included a total of 15 patients, 8 females (53.3%) and 7 males (46.7%), with different clinicopathological characteristics. The most frequent clinical condition inducing aHSCT was acute lymphocytic leukemia (40.0%). Conditioning regimens consisted primarily in busulfan and cyclophosphamide, with anti-thymocyte globulin, and myeloablation was the preferential treatment. Epstein-Barr virus (EBV) was present in all patients with a median time of diagnosis following transplant of 75 days (range, 25-485 days) and a median viral load of 4.75 $\log_{10}$ copies/ml (range, 3.30-6.26 $\log_{10}$ copies/ml). PTLD diagnosis was mainly assessed by clinical findings, and histological confirmation was available for 5 patients: 3 monomorphic, 1 polymorphic and 1 with early lesions of PTLD. To the best of our knowledge, this is the first study to describe PTLD cases in HSCT patients in Portugal. The data reinforces the importance of performing EBV monitoring in high-risk patients, particularly those receiving a transplant from mismatch/unrelated donors, and those with myeloablative conditioning regimen including antithymocyte globulin. The results also suggested that EBV viral load may be significant for the prediction of PTLD development.

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Key words: post-transplant lymphoproliferative disorder, post-transplant lymphoproliferative disease, Epstein-Barr virus

Introduction

The development of lymphoid proliferations after transplantation has been recognized for more than a quarter of century as an important morbidity factor (1). The post-transplant lymphoproliferative disorder (PTLD) refers to a heterogeneous group of lymphoproliferative diseases, which vary from uncomplicated, self-limiting infectious mononucleosis, to malignant lymphoma. The histological characterization varies from reactive-appearing, polyclonal lymphoid infiltrates or undifferentiated cells that are morphologically indistinguishable from malignant lymphoma or plasma cell myeloma (2-4).

PTLD is relatively rare; nevertheless, it is the most frequent malignant disease early after transplantation, with the majority of cases being reported in the first year after transplantation (3,5,6). Risk factors for PTLD development include young age and age over 50 years at transplantation, white race, unrelated or HLA-mismatched graft, Epstein-Barr virus (EBV)-seronegative status prior to transplant, primary EBV infection, type of organ transplant, intensity of immunosuppression and the occurrence of concomitant cytomegalovirus disease (3,7).

Not all PTLD cases are EBV-related, but consistent data recognize primary EBV infection as the most important risk factor for PTLD development (8,9). Indeed, the immunosuppression after transplantation in an EBV-seropositive

patient reduces the activity of the patients' EBV-specific cytotoxic T-cell surveillance, which increases the probability of uncontrolled proliferation of EBV-infected B-cells and subsequent progression to PTLD (10). Moreover, transplant recipients experiencing primary EBV infection during the early post-transplant period seem to be particularly susceptible to develop PTLD of B-cell origin, reflecting their lack of any preexisting EBV-specific T-cell immunity (3,10).

The overall incidence of PTLD varies from 1 to 22% depending on the presence of risk factors, namely the transplanted organ, patient age, EBV serostatus from recipient and donor, aggressiveness of immunosuppression (11). The cumulative incidence of PTLD in allogeneic hematopoietic stem cell transplantation (aHSCT) recipients is approximately 1.0% (range 0.5-1.8%), with slightly higher rates in the pediatric population (1,12). Survival rates depend mainly on the type of PTLD, extent of disease and patient age: While pediatric patients and those with localized disease seem to have a better prognosis, monomorphic PTLDs are the most aggressive forms (5,7,13).

The purpose of this study was to examine the clinical and pathologic characteristics, as well as the outcome of PTLD after aHSCT, in patients diagnosed at the Portuguese Oncology Institute of Porto (Porto, Portugal) between 2005 and 2012.

Materials and methods

Type of study and study participants. We retrospectively reviewed the clinic-pathological and EBV infection data of patients that developed PTLD after aHSCT at the Bone Marrow Transplantation Unit from Portuguese Oncology Institute of Porto in 2005 and 2012. This retrospective study was approved by the Ethical Committee of IPO Porto. The present study included a total of 15 patients, 8 females (53.3%) and 7 males (46.7%). When available, cases were histologically confirmed by an expert pathologist and classified according the most recently available edition of the World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues (4th edition).

Sample processing and EBV detection. Samples were collected in EDTA-containing tubes (Vacutainer®; BD Biosciences, Franklin Lakes, NJ, USA) and stored in freezing temperature prior to processing. Blood samples were collected retrospectively from the institution archives. DNA was extracted by MagNA Pure Compact Nucleic Acid Isolation kit I (Roche Diagnostics GmbH, Mannheim, Germany). DNA/RNA quality was assessed by measuring the absorbance at 260/280 nm using the NanoDrop 1000 Spectrophotometer v3.7 (Thermo Fisher Scientific, Inc., Wilmington, DE, USA).

All patients submitted to aHSCT were monitored for EBV infection upon request from clinicians after clinical suspicion. EBV detection was performed at the Virology Service of IPO Porto using the commercial Real-Time PCR kit EBV Q-PCR Alert (Nanogen Advanced Diagnostics S.p.A., Trezzano sul Naviglio, Italy) which targets a region from EBV nuclear antigen 1 gene (EBNA1). Amplification was performed with the ABI PRISM 7300 Sequencer Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and results were obtained by measuring the geometric increase of probe fluorescence during amplification and samples were considered positive when the exponential curve exceeded the cycle threshold line.

Regarding amplification quality, positive and negative controls were used: as negative control we used double distilled water in replacement of template DNA; and as positive control we have used samples from the external quality control panel used at the Virology Service for EBV diagnosis.

Data collection. Clinic-pathological data was extracted from institutional databases including pre-transplant recipient age, gender, underlying disease, HLA-donor-recipient status, EBV serological status of the recipient, source of stem cells, conditioning regimen and use of ATG; post-transplant information (clinical findings, date of PTLD suspicion, date of PTLD confirmation, PTLD type, GVHD prophylaxis, GVHD type and outcome) and viral data (date of EBV suspicion, EBV viral load).

Statistical analysis. Statistical analysis was performed using the SPSS version 20.0 software (IBM Corp., Armonk, NY, USA). Overall survival was defined as the time between the date of transplant and the date of last follow-up or mortality. The differences in survival were calculated using the log-rank test and the Kaplan-Meier method.

Table I. Clinical characteristics of patients.

Variable	n (%)
Age, median (range); years	10 (3-38)
Sex	
Male	7 (46.7)
Female	8 (53.3)
Underlying disease	
Acute leukemia	10 (66.6)
Chronic leukemia	1 (6.7)
Myelodysplastic/myeloproliferative syndrome	1 (6.7)
Others	3 (20.0)
HLA donor	
Match/related	4 (26.7)
Mismatched/unrelated	11 (73.3)
Source of cells	
PBSC	12 (80.0)
BM	2 (13.3)
UCB	1 (6.7)
Conditioning regimen	
MAC	14 (93.3)
RIC	1 (6.7)
ATG	
Yes	12 (85.7)
No	2 (14.5)

ATG, anti-thymocyte globulin; PBSC, peripheral blood stem cells; BM, bone marrow; UCB, umbilical cord blood; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; HLA, human leukocyte antigen.

Table II. Transplant-associated patient information.

Patient	Age (years)	Gender	Diagnosis	Pre-conditioning	ATG	Myeloablation	Donor	Source	GVHD prophylaxis	GVHD type	Outcome
1	25	Male	PNH	BuCy2ATG	Yes	Yes	UMD	PB	Tacrolimus	Acute	Mortality
2	6	Male	PI	Bu12Cy2ATG	Yes	Yes	UMD	BM	Tacrolimus+MTX	Acute	Alive
3	6	Male	ALL	Bu12Cy120MelfATG	Yes	Yes	UMD	PB	Tacrolimus+MTX	Acute/chronic	Alive
4	38	Female	ALL	Bu12Cy120MelfATG	Yes	Yes	UMD	PB	CSP+MTX	Chronic	Alive
5	16	Male	ALL	Bu12Cy120MelfATG	Yes	Yes	UMD	PB	Tacrolimus+MTX	Acute/chronic	Mortality
6	23	Female	ALL	BuCyMelfATG	Yes	Yes	UMD	PB	Tacrolimus+MTX	Acute	Mortality
7	8	Male	ALL	BuCyMelfATG	Yes	Yes	UMD	PB	Tacrolimus+MTX	Acute/chronic	Mortality
8	28	Male	AML	BuCy2ATG	Yes	Yes	UMD	PB	Tacrolimus+MTX	Acute/chronic	Alive
9	10	Male	AML	Bu12Cy2ATG	Yes	Yes	UMD	PB	Tacrolimus+MTX	Chronic	Mortality
10	36	Female	AML	BuCy2ATG	Yes	Yes	MRD	PB	Tacrolimus+MTX	Acute/chronic	Alive
11	6	Female	AML	Bu16Cy4ATG	Yes	Yes	UMD	BM	Tacrolimus+MTX	Acute/chronic	Alive
12	3	Female	CML	BuCyATG	Yes	Yes	UMD	UCB	Tacrolimus	NA	Alive
13	19	Female	MDS	BuCy2ATG	Yes	Yes	UMD	PB	Tacrolimus+MTX	Acute/chronic	Mortality
14	4	Female	CAT	AlemtuzumabFluCy	Yes	Reduced intensity	UMD	PB	T-cell depletion	NA	Mortality
15	8	Female	ALL	BuCyMelf	No	Yes	UMD	PB	Tacrolimus+MTX	Acute/chronic	Alive

PNH, paroxysmal nocturnal hemoglobinuria; PI, primary immunodeficiency; ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic/myeloproliferative syndrome; CAT, congenital amegakaryocytic thrombocytopenia; Bu, busulfan; Flu, fludarabine; Cy, cyclophosphamide; ATG, antithymocyte globulin; Melf, melphalan; PB, peripheral blood; BM, bone marrow; UCB, umbilical cord blood; MTX, methotrexate; CSP, cyclosporine; UMD, unrelated/mismatch donor; MRD, match/related donor; NA, not available; GVHD, graft-versus-host disease.

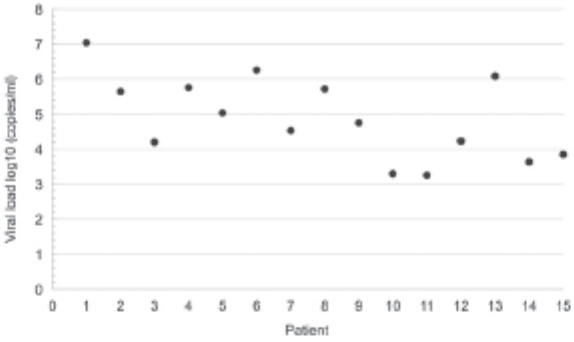

Figure 1. Viral load distribution from all patients involved in the retrospective study.

Results

The study included a total of 15 patients, 8 females (53.3%) and 7 males (46.7%), with median age of 10 years-old (range 3-38)- Table I. Patients had a median follow-up time of 14 months (range: 2-72). Primary diagnoses of patients included in this study included paroxysmal nocturnal hemoglobinuria (n=1), primary immunodeficiency (n=1), acute lymphocytic leukemia (n=6), acute myelogenous leukemia (n=4), chronic myelogenous leukemia (n=1), myelodysplastic/myeloproliferative syndrome (n=1) and congenital amegakaryocytic thrombocytopenia (n=1). Most of patients had mismatched/unrelated donors (73.3%) and the collection of cells was mainly performed by peripheral blood stem cells (80.0%). Myeloablative conditioning was used in 14 patients and ATG in 12 patients. Transplant-related information for each patient is described in Table II.

Regarding the clinical presentation of patients, 2 presented with fever, 12 had increased liver enzymes, adenomegalies were observed in 2 patients and 12 patients had also increased lactate dehydrogenase. EBV serological status prior to transplantation were evaluated according to presence of IgM and IgG titers in plasma samples. Serological status was divided in three groups: susceptible (absence of IgM and IgG), active infection (presence of IgM and/or IgG) and finally, past infection (absence of IgM and presence of IgG).

The development of EBV infection was present in all of 15 patients, with a median time of diagnosis after transplant of 68 days (range 29-464 days), with 80% (n=12) of them detected <180 days after transplant, and with a median viral load of 4.75 $\log_{10}$ copies/ml (range 3.30-6.26 $\log_{10}$ copies/ml; Fig. 1). PTLD diagnosis occurred approximately in the same period where EBV infection occurred (mean 135, median 75 days and range 25-485 days vs. mean 130 days, median 68 days and range 29-464 days, respectively). PTLD classification was available for only 5 patients and included monomorphic-type PTLD (n=3), polymorphic PTLD (n=1) and reactive plasmocytic hyperplasia (early lesions) (n=1) (Table III; Fig. 2). The remaining cases were not histological confirmed, and diagnosis was established by considering all clinical findings.

We observed graft-vs.-host disease (GVHD) in 13 patients (93.3%): 3 with acute GVHD (20.0%), 2 with chronic GVHD (13.3%) and 8 with both (53.3%). Considering the grade of acute GVHD, all patients with clinical information had a grade of II or higher. Regarding chronic GVHD, 3 patients had an evolution of acute-to-chronic, while 7 had a *de novo* chronic GVHD; two patients experienced extensive disease and 5 had only limited disease (Table II).

As for the overall outcome, 8 patients are still alive (53.3%), 5 with no signs of disease (33.3%) and 3 with evidence of disease (20.0%); and 7 patients have died (46.7%), 4 from complications associated with the transplant (26.7%) and 3 from progression of disease (20.0%) (Table II). A Kaplan-Meier plot was obtained by evaluating the cumulative survival of these patients, in months (Fig. 3).

Discussion

PTLD is one of the most serious complications of immunosuppression in patients who undergo hematopoietic stem cell transplantation, with high impact on morbidity and mortality (14). EBV infection has been strongly associated with the development of PTLD, and this association is widely described in the literature (15,16).

In this retrospective analysis, we verified that PTLD affects individuals of all age groups and with several types of hematological malignancies, the majority having had unrelated donors. Our patients had different types of pre-conditioning regimens (myeloablative in 14 patients), with predominance of busulfan and cyclophosphamide. Since types of regimens are varied, they appear not to have a direct correlation with the development of PTLD. ATG was used in almost all patients except for two, and without absolute prevalence date it is difficult to confirm if its use is directly correlated with PTLD development. GVHD prophylaxis was performed mainly with tacrolimus, and concomitant with MTX, and still patients have developed some type of GVHD which indicates that altering prophylaxis regimen should be taken into consideration.

In our case series, EBV infection was diagnosed at a median of 68 days after transplant. EBV infection is frequently associated with the intermediate period after aHSCT, mainly

Table III. Characteristics of PTLD and EBV in patients.

Patient	Age (years)	Gender	Diagnosis	Clinical findings	EBV IgM	EBV IgG	EBV serostatus	TT EBV infection (days)	Viral load (copies/ml)	Viral load (Log copies/ml)	PTLD classification	Biopsy vs. excision (topography)
1	25	Male	PNH	Fever, Adenomegaly, Hepatomegaly; ↑ liver enzymes and LDH	NR	R	Past infection	53	1.10×10^7	5.64	NA	NA
2	6	Male	PI	Fever; ↑ liver enzymes	NR	R	Past infection	29	4.40×10^5	4.20	NA	NA
3	6	Male	ALL	↑ liver enzymes	NR	R	Past infection	123	1.60×10^4	5.76	NA	NA
4	38	Female	ALL	↑ liver enzymes and LDH	NR	R	Past infection	38	5.70×10^5	5.04	NA	NA
5	16	Male	ALL	↑ liver enzymes and LDH	R	R	Active infection	150	1.10×10^5	6.26	Monomorphic (Case 2)	Biopsy (amygdala)
6	23	Female	ALL	↑ liver enzymes and LDH	NR	R	Past infection	68	1.80×10^6	4.53	Monomorphic (Case 3)	Biopsy (amygdala)
7	8	Male	ALL	↑ liver enzymes and LDH	NR	R	Past infection	46	3.40×10^4	5.72	NA	NA
8	28	Male	AML	Adenomegaly; Pancytopenia; ↑ liver enzymes and LDH	NR	R	Past infection	53	5.30×10^5	4.75	Monomorphic (Case 1)	Excision (Cervical lymph node)
9	10	Male	AML	↑ liver enzymes	NR	R	Past infection	60	5.60×10^4	3.30	NA	NA
10	36	Female	AML	↑ LDH	NR	R	Past infection	333	2.00×10^3	3.26	Early lesions (Case 5)	Excision (Lymph node)
11	6	Female	AML	↑ liver enzymes and LDH	NR	R	Past infection	285	1.80×10^3	4.23	NA	NA
12	3	Female	CML	↑ liver enzymes and LDH	NR	R	Past infection	464	1.70×10^4	6.08	Polymorphic (Case 4)	Excision (Cervical lymph node)
13	19	Female	MDS	↑ LDH	NR	R	Past infection	44	1.20×10^6	3.63	NA	NA
14	4	Female	CAT	↑ LDH	NR	R	Past infection	82	4.30×10^3	5.64	NA	NA
15	8	Female	ALL	↑ liver enzymes and LDH	NR	NR	Susceptible	119	7.00×10^3	3.85	NA	NA

PNH, paroxysmal nocturnal hemoglobinuria; PI, primary immunodeficiency; ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic/myeloproliferative syndrome; CAT, congenital amegakaryocytic thrombocytopenia; LDH, lactate dehydrogenase; NR, non-reactive; R, reactive; TT, time to; NA, not available; EBV, Epstein-Barr virus; PTLD, post-transplant lymphoproliferative disorder.

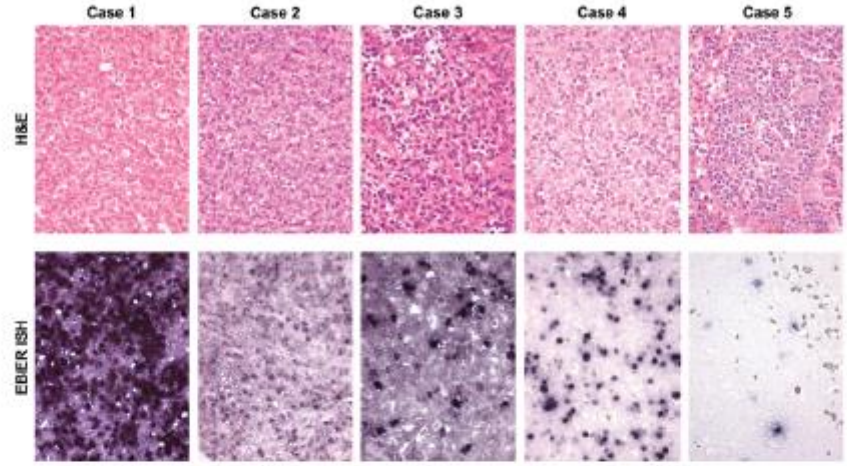

Figure 2. H&E staining for post-transplant lymphoproliferative disorder diagnosis and classification (magnification, x400). H&E, hematoxylin and eosin; EBER ISH, Epstein-Barr virus-encoded RNA *in situ* hybridization.

between 3 weeks to 3 months after transplant (17). Viral infection during this period is correlated with delayed or incomplete reconstitution of specific immunity, or patients experiencing GVHD (18). Regarding PTLD, frequently, the median onset of development is 3 months, with a range of 2-5 months after transplantation (13), which is consistent with our data. Symptoms are quite nonspecific, with patients presenting with fever, malaise, enlarged lymph nodes and high levels of LDH, which were the factors for clinical PTLD suspicion in our patients (2). All patients that developed PTLD had an EBV infection at some point after transplantation. EBV positivity is directly related to PTLD development since its infection, or increase in viral load up to 2,000 copies/ml, occurs mainly at the same time PTLD is diagnosed. PTLD is more frequent in EBV-seronegative patients receiving allografts from EBV-seropositive donors and in patients with delayed immune reconstitution due to T-cell-depletion or HLA-mismatched donor. In a study conducted by Brunstein *et al* (19), 15 of 335 patients developed a EBV-related complication, at a median of 133 days (range 52-407 days), which is consistent with our results.

As previously described by Bhatia *et al* (20), PTLD has mortality rates reaching up to 70-90%, which is higher than our results (46.7%). Survival rates depend on age and stage of disease at the time of diagnosis, with pediatric and patients with localized disease showing the best prognosis (5). In our study, overall patient survival was not affected by the development of PTLD.

This is the first study to describe PTLD cases in HSCT patients in Portugal, combining data from several years at a reference transplantation center. This study demonstrates that EBV infection occurs mainly between 2 and 4 months after transplant and precedes the development of PTLD, and especially the viral load may be important for the monitorization and early diagnosis of PTLD. Thus, the study shows the importance of identify high-risk patients for PTLD development and

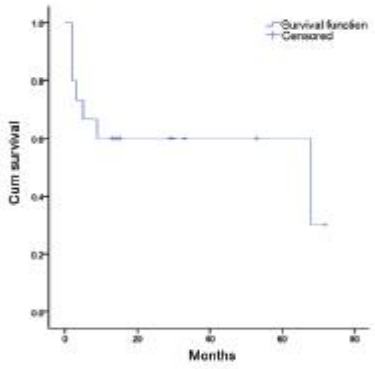

Figure 3. Kaplan-Meier plot for survival analysis of patients with post-transplant lymphoproliferative disorder. Cum, cumulative.

to provide them a frequent monitorization of EBV viral load as suggested by recent guidelines (21,22).

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JMD and HS designed the study, JL and RH performed the histological analysis of cases, and CPV, LR, RB, FC and ACJr revised the clinical information obtained from patients. IB and RM provided the laboratory data, and JMD and HS performed data analysis and drafted the manuscript. All authors read and revised the manuscript.

Ethics approval and consent to participate

This retrospective study was approved by The Ethics Committee of Portuguese Oncology Institute of Porto (Porto, Portugal). The need for written informed consent was waived due to the retrospective nature of the study.

Consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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ATTACHMENT II – Journal Article (Study II)

Association of Epstein-Barr virus infection with allogeneic hematopoietic stem cell transplantation in patients in Portugal

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Abstract. The identification of patients at higher risk of developing Epstein-Barr virus (EBV) infection in hematopoietic stem cell transplants (HSCT) is useful for the prevention of EBV-associated diseases A prospective observational study was developed that included 40 patients (27 male and 13 females, with mean age of 32.2±1.5 years old) undergoing allogeneic-HSCT between January and December 2015. EBV was examined in whole blood samples collected during routine procedures at day (D)+30, D +60, +90, D+120, D+150 and D+180 post-transplant. EBV was detected, at least once during the follow-up period in 70.0% of our patients. Results indicated that patients with unrelated donors had increased risk of developing EBV infection at D+60 and D+150 (OR=3.9, P=0.058; OR=8.0, P=0.043; respectively). Moreover, myeloablative conditioning (OR=4.3, P=0.052), anti-thymocyte globulin use (OR=12.0, P=0.030) and graft-vs.-host disease (OR=6.7, P=0.032) were associated with EBV infection at D+60, D+150 and D+90, respectively. In our series, none of these patients developed post-transplant lymphoproliferative disease. To the best of our knowledge, the present study is the first study to report EBV infection in patients undergoing aHSCT from Portugal. The study revealed that EBV infection is associated with different factors. These findings provide evidence towards the identification of high-risk patients for EBV-infection and associated disease.

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Key words: Epstein-Barr virus, hematopoietic stem cell transplant, post-transplant lymphoproliferative disease, graft-vs.-host disease

Introduction

Hematopoietic stem cell transplants (HSCT) is an effective therapy in the treatment of hematological malignancies such as leukemias and lymphomas (1). The regimens required for transplant produce profound immune deficiency in the early period after transplantation (2). Conditioning regimens include: Myeloablative (MAC) and reduced intensity conditioning (RIC). MACs include concomitant or single use of alkylating agents, such as cyclophosphamide (Cy) and busulfan (Bu), while RICs are mainly performed with fludarabine (Flu) or low doses of total body irradiation (TBI) (3,4). Choice of conditioning depends on patient's age, underlying disease, relevant comorbidities and type of donor. These regimens are related to several risks such as infections, graft-vs.-host disease (GVHD) and post-transplant lymphoproliferative disorder (PTLD) (5). GVHD occurs in approximately 40-90% of transplanted patients (6). ATG seems to be effective in GVHD prophylaxis, and is related to reduced rates of relapses and infections in adults who undergo bone marrow (BM) or peripheral blood stem cells (PBSC) transplant (7).

Rates of mortality after HSCT often reach up to 50.0% (5). Some studies describe that intensive conditioning regimens are associated with reduction of tumor relapses, although it might simultaneously increase the transplant-related mortality rates, including the mortality of infections (4).

Epstein-Barr virus (EBV) is a ubiquitous Human Herpes virus and infects 50-89% of children and remains latent, in memory B cells, of ~90% of adults (8). Viral infections are known to be a major cause of morbidity and mortality in patients undergoing HSCT and Herpes virus are known to be among the most common viral infections in these patients (5). The iatrogenic suppression of T-cell with the immunosuppression of the transplant regimens, allows the proliferation of infected B cells (9). EBV is one of the most important viruses in transplanted patients, and monitoring of EBV DNA in peripheral blood is routinely performed in several transplant centers, since these patients have a higher-risk of developing complications (9-13).

This study aims to analyze the impact of the different characteristics of allogeneic-HSCT (aHSCT) patients and correlate with the development of EBV infection.

Materials and methods

Type of study and study participant. A prospective follow-up study was performed with 40 consecutive patients who underwent aHSCT at the *Bone Marrow Transplant Service* of *Portuguese Oncology Institute of Porto* (IPO Porto; Porto, Portugal) between January and December of 2015. Cases were selected randomly from the cohort of patients undergoing aHSCT. The study was approved by the local Ethical Committee and did not interfere with the routine procedures decided by clinicians. Clinical data was collected from individual clinical records and stored in a database with unique codification.

Sample processing. EBV detection is not routinely requested for all HSCT patients in our institution (only for high-risk), and therefore we have used samples collected during routine procedures for patients monitoring selected at 6 different times: D+30, +60, +90, +120, +150 and +180 days after transplant. Samples were collected in EDTA-containing tubes and stored prior to processing.

Sample processing was performed at the Virology Service of IPO Porto. DNA was extracted by *MagNA Pure Compact Nucleic Acid Isolation kit I* (Roche, Germany). DNA/RNA quality was assessed by measuring the absorbance at 260/280 nm with NanoDrop 1000 Spectrophotometer v3.7 (Thermo Fischer Scientific, Inc., Wilmington, MA, USA).

EBV detection. EBV detection was performed with a real-time PCR protocol targeting EBV polymerase gene (EBV POL) as previously reported (14). Amplification was performed with the ABI PRISM 7300 Sequencer Detection System (Applied Biosystems, Foster City, CA, USA) and results were obtained by measuring the geometric increase of probe fluorescence during amplification and samples were considered positive when the exponential curve exceeded the cycle threshold line. All amplifications used positive and negative controls: As negative control, we used double distilled water in replacement of template DNA; and as positive control we have used samples from the External Quality Control panel for EBV used at the Virology Service. Results were independently analyzed by two of the authors and 10% of all samples were randomly selected and re-submitted to amplification to confirm the results.

Statistical analysis. Statistical analysis was performed with IBM® SPSS Statistics 20 software (IBM Corp., Armonk, NY, USA) for Mac. Chi-square or Fisher's exact test with a 5% significance level were used to estimate odds ratio (OR) and the corresponding 95% confidence intervals (CIs) as a measure of association between the categorical variables and the risk of EBV infection. Cox proportional hazard models were used to assess the risk factors associated EBV infection. Kaplan-Meier with log-Rank test was used to calculate the association between EBV infection and post-transplant survival (OS).

Results

Clinical characteristics. This study included 40 patients, 27 males (67.5%) and 13 females (32.5%), with ages between 1 and 63 years-old (mean 32.2±1.5, median 35 years old) who underwent aHSCT at our institution between January and December of 2015.

Table I. Clinical characteristics of patients.

Characteristics	Number (%)
Age, median (range) years old	32.2 (1-63)
Sex, n (%)	
Male	27 (67.5)
Female	13 (32.5)
Underlying disease, n (%)	
Aplastic anemia	3 (7.5)
Acute leucemia	23 (57.5)
Chronic leucemia	2 (5.0)
Non-Hodgkin lymphoma	1 (2.5)
Multiple myeloma	1 (2.5)
Myelodysplastic/myeloproliferative syndrome	7 (17.5)
Others	3 (7.5)
Conditioning regimen, n (%)	
BuCy	4 (10.0)
BuCy2	20 (50.0)
Cy	1 (2.5)
FluBu	10 (25.0)
FluCy	3 (7.5)
FluMelf	1 (2.5)
ATG, n (%)	
Yes	20 (50.0)
No	20 (50.0)
Type of donor, n (%)	
Related	19 (47.5)
Mismatched/unrelated	21 (52.5)
Source of cells, n (%)	
PBSC	33 (82.5)
BM	5 (12.5)
UCB	2 (5.0)

Bu, busulfan; Cy, cyclophosphamide; Flu, fludarabine; Melf, Melphalan; ATG, anti-thymocyte globulin; PBSC, peripheral blood stem cells; BM, bone marrow; UCB, umbilical cord blood.

Table I demonstrates the characteristics of all patients. Briefly, patients were submitted to aHSCT due to different hematological malignancies, including aplastic anemia (n=3), acute leukemia (n=23), chronic leukemia (n=2), non-Hodgkin lymphoma (n=1), multiple myeloma (n=1), myelodysplastic/myeloproliferative syndrome (n=7) and others, including primary immunodeficiency, myelofibrosis and severe combined immunodeficiency (n=3). Off the 40 patients submitted to aHSCT only one was being transplanted for the second time. When evaluating the donor-receptor relation, 21 patients had unrelated donors (52.5%) and the remaining 19 had related donors (47.5%). Regarding the HLA-match, only one patient received a graft from a HLA-mismatched donor. The source of cells for transplant was mainly from peripheral blood (82.0%), while the remaining were from BM (13.0%) and umbilical cord blood (5.0%). MAC

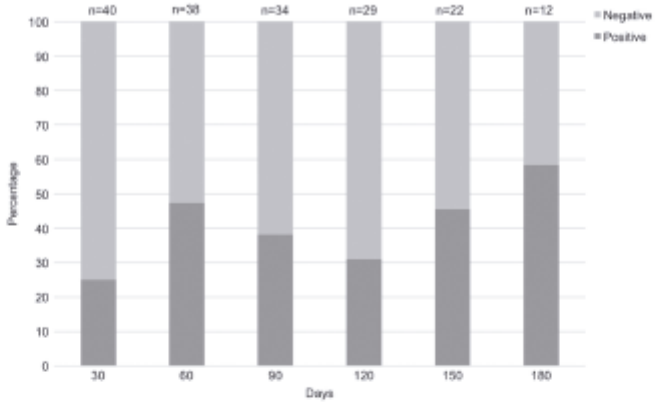

Figure 1. EBV infection at different times during post-transplant monitoring. EBV, Epstein-Barr virus.

conditioning was used in 24 (60.0%) of our patients, with Bu and cyclophosphamide, as well as ATG which was used in 14 of these patients. Reduced intensity regimens were used in 16 patients with 6 of them receiving ATG. Patients with unrelated donors, except in one case, received ATG as part of GVHD prophylaxis. Prophylaxis for GVHD was performed for all patients (data not available for 2 patients). Acute GVHD (aGVHD) was observed in 21 patients, all of them with grade 2 or higher; while chronic GVHD (cGVHD) was present in 5 patients, 4 with evolution from aGVHD and only one with *de novo* cGVHD.

EBV and CMV serological status was described as: IgM positive and IgG negative/positive (active infection); IgM negative and IgG positive (past-infection); and IgM negative and IgG negative (susceptible). In our study, we found 3 patients were susceptible for EBV-primary infection, 1 patient had an active EBV-infection and 7 patients were susceptible of CMV-primary infection (data not shown).

EBV infection. The overall data reveal that 70% of patients were at least once positive during the follow-up period. Regarding the specific times of sample collection in our study, results showed the presence of EBV in 10/40 (25%) at D+30, 18/38 (47%) at D+60, 13/34 (38%) at D+90, 9/29 (31%) at D+120, 10/22 (45%) at D+150, and 7/12 (58%) at D+180 (Fig. 1).

The analysis of EBV DNA positivity (at least once positive) and its association with clinicopathological characteristics of patients is shown in Table II. The analysis according to sex seem to show that post-transplant EBV infection is more frequent in females (OR=8.33, P=0.033). Despite not statistically significant, we have found that EBV infection is more frequent in patients with unrelated donors (OR=3.12, P=0.112), engrafted with PBSC (OR=2.00, P=0.414), submitted to MAC conditioning regimen (OR=2.96, P=0.121), using ATG in the conditioning regimen (OR=2.91, P=0.135), and that developed aGVHD (OR=3.09, P=0.112). Despite we observe an increased prevalence of EBV in older patients, there was no statistically significant association regarding age (child vs. adults, or median age) (P>0.050). EBV serostatus prior to transplant does not seem to be related with the development of infection during the post-transplant period (data not shown).

The analysis of EBV infection at the different times during the follow-up period revealed different associations with clinicopathological data, despite not all with statistical significance. At D+60, EBV infection was associated with transplants from unrelated donors (OR=3.9, P=0.058), with MAC conditioning (OR=4.3, P=0.052) and use of ATG (OR=3.6, P=0.099), although, with no statistical significance; at D+90, development of GVHD was related a higher risk of infection (OR=6.7, P=0.032); and finally, at D+150, EBV infection was associated with unrelated donors (OR=8.0, P=0.043), use of ATG (OR=12.0, P=0.030) and development of GVHD (OR=5.6, P=0.099). There was no significant association between age and EBV infection in different times (data not shown). The Cox regression analysis considering patients sex, type of donor, conditioning regimen, use of ATG and development of aGVHD, revealed an association of unrelated donor with EBV infection at D+150 (HR=8.8, P=0.030).

Follow-up. None of these patients had clinical suspicion or development PTLD during follow-up. Of the 40 patients included in this study, 16 patients have deceased, 6 are alive with evidence of disease and 18 are alive without evidence of disease. Cumulative survival was evaluated by performing a Kaplan-Meier plot and estimated survival time was approximately 476±58.7 days (data not shown).

Analysis of EBV-infection impact on overall survival (OS) is shown in Fig. 2. The impact of EBV infectionon OS, in different times, is shown in Fig. 3. Results suggest that EBV positivity at D+90 days and D+180 may be associated with increased mortality (P=0.095, 303.3 vs. 593.2 days and P=0.097, 367.0 vs. 679.5, respectively).

Discussion

aHSCT is an option for the treatment of hematological malignancies and these patients are submitted to pre-transplant treatments that reduce significantly the immune system to avoid rejection

Table II. Analysis of EBV infection among allogeneic hematological stem cell transplant recipients.

Variable	EBV infection n (%)	P-value	OR (95% CI)
Sex			
Male (n=27)	16 (59.3)	0.033	8.33 (0.93-100)
Female (n=13)	12 (92.3)		
Stem cell source			
Cord blood or bone marrow (n=7)	4 (57.1)	0.414	2.00 (0.37-11.1)
Peripheral blood (n=33)	24 (72.7)		
Conditioning regimen			
Reduced intensity (n=16)	9 (56.3)	0.121	2.96 (0.73-11.9)
Myeloablative (n=24)	19 (79.2)		
ATG			
With (n=19)	11 (57.9)	0.135	2.91 (0.70-12.1)
Without (n=20)	16 (80.0)		
Donor			
Related (n=19)	11 (57.9)	0.112	3.12 (0.75-12.5)
Unrelated (n=21)	17 (81.0)		
Acute GVHD			
Absent (n=19)	11 (57.9)	0.170	3.09 (0.75-12.8)
Present (n=21)	17 (81.0)		
Age			
<20 years old (n=14)	8 (57.1)	0.173	2.50 (0.62-10.1)
≥20 years old (n=26)	20 (76.9)		
<35 years old (n=20)	13 (65.0)	0.366	1.61 (0.41-6.34)
≥35 years old (n=20)	15 (75.0)		

ATG, anti-thymocyte globulin; GVHD, graft-vs.-host disease; CI, confidence interval; OR, odds ratio; EBV, Epstein-Barr virus.

of the graft (15). This immunosuppression is associated with the occurrence of different events in the post-transplant period, such as development of GVHD, infections and PTLDs (16). Viral infections are a major concern in the subset of aHSCT, and while CMV infection has been consistently associated with a significant morbidity/mortality increase (17-19), EBV infection has been underestimated in these patients since only a minority will suffer from EBV-associated complications, such as PTLD (11,13,20).

The incidence of EBV DNAemia varies within transplant centers, ranging from 0.1 to 63% (11,21,22). In our study, we verified that 70.0% of our patients were positive for EBV, at least once during the follow-up period, nevertheless the prevalence of EBV-positive patients at a specific time ranged from 25-58%. Dumas *et al*, monitored EBV viral load at least once a week for 3 months and verified that EBV DNAemia occurred in approximately 14.0% of patients, which is significant lower than what we describe (23). Hence, these results show that there are significant differences amongst transplant centers that may be explored considering the individual characteristics of patients.

Literature refers that EBV infection is most common within the first 100 days post-transplant, in high-risk patients (24,25). In our study, we describe that EBV infection varied throughout the follow-up period, with a mean of 65.6±39.6 days (range, 27-183). According to the guidelines for diagnosis and monitoring of EBV DNAemia, the monitoring should be performed by quantitative PCR and monitorization must start at the first month post-transplant, and should be performed at least during 4 months after transplantation, with

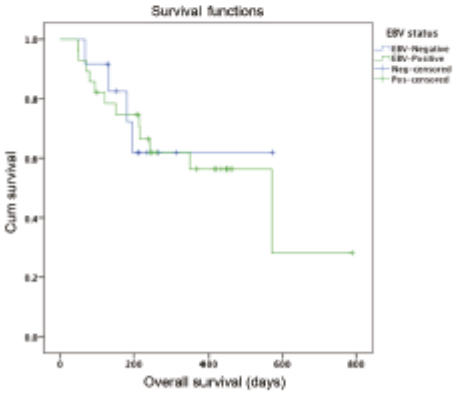

Figure 2. Kaplan-Meier plots with log-rank test estimate the post-transplant survival of aHSCT patients with and without EBV infection. aHSCT, allogeneic-hematopoietic stem cell transplant; EBV, Epstein-Barr virus.

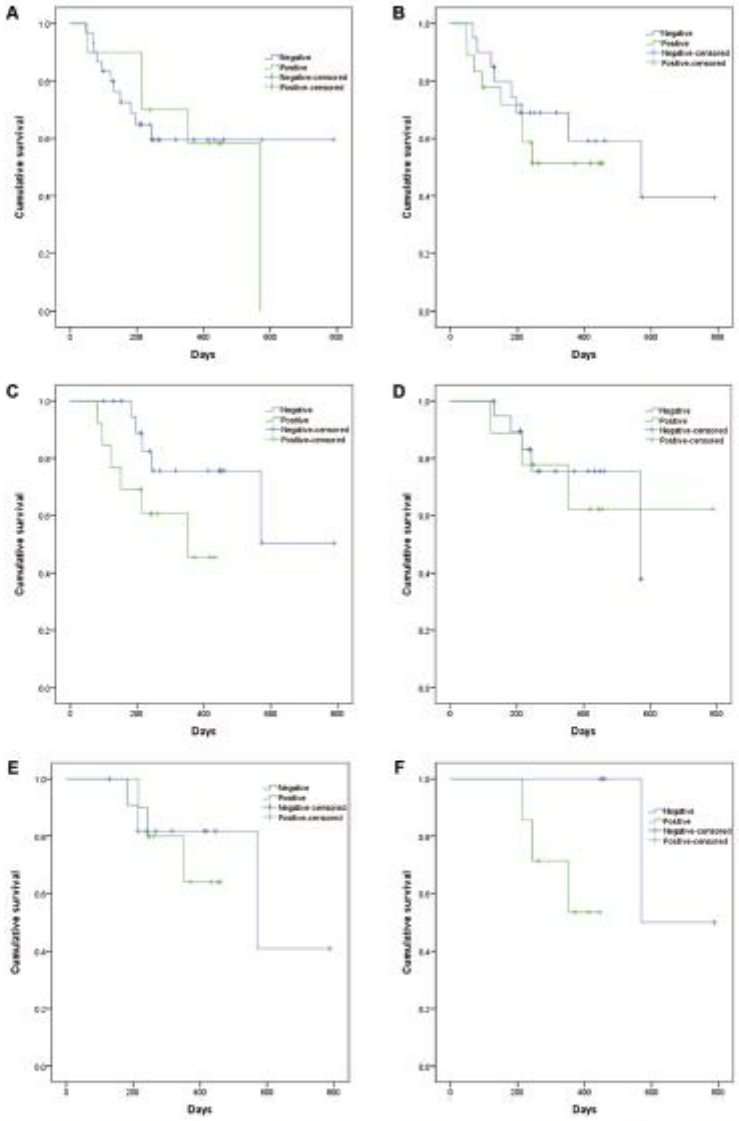

Figure 3. Kaplan-Meier plots with log-rank test estimate the post-transplant survival of aHSCT patients with and without EBV infection at the different times after transplantation. (A) D+30 post-transplant, (B) D+60 post-transplant, (C) D+90 post-transplant, (D) D+120 post-transplant, (E) D+150 post-transplant, and (F) D+180 days post-transplant. aHSCT, allogeneic-hematopoietic stem cell transplant; D, day; EBV, Epstein-Barr virus.

a weekly frequency (11,12). Despite this, many authors discuss the cost-effectiveness of EBV monitoring once-a-week and therefore many studies are required to show what would be the best time and interval of monitoring. Moreover, EBV viral load has been having different input data since the cutoff viral loads for treatment are variable and the development of international standardization of EBV viral load management is yet to be defined (12,13,26). Gulley and Tang, affirm that routine monitoring of EBV infection is viable in PTLD prevention, although further studies must be done to correlate specific viral

loads in the identification of high-risk patients (27). As defined by published guidelines, rituximab therapy is used prophylactically before or shortly after transplant to reduce the risk of EBV DNAemia and PTLD development in high-risk patients, such as patients with EBV-seropositive donors (11). In these cases, viral load is also informative, when there is a withdrawal of immunosuppression, and rituximab administration, to verify if the treatment is being successful (27).

The identification of high-risk patients for EBV infection is crucial for the correct clinical approach of EBV-associated morbidity/mortality in aHSCT patients. Accessing variables associated with transplant, and complications associated with treatment, such as PTLD, include patients into two groups: High-risk and low-risk. High risk patients have pre-transplant risk factor such as T-cell depletion, EBV serology donor/recipient mismatched, cord blood transplantation, HLA mismatch, Splenectomy and second HSCT; and post-transplant risk factors as severe acute or cGVHD and high or rising EBV DNAemia (11,13,25,28).

Our data showed that patients with unrelated and/or mismatched donors were more prone to develop an EBV infection post-transplant, and that using peripheral blood as source of stem cells adds a higher risk for EBV infection. Studies report EBV infections of 8.8% in MAC conditionings and 35.0% in RICs (29), 54.0% in T-cell depletion (30) and 65.0% in T-cell depletion concomitant with ATG use (22). Xuan *et al* showed that the use of ATG, HLA-mismatched, unrelated donor and acute aGVHD were identified as risk factors for EBV infection (4). These findings are according those reporting that intensified conditioning increase the incidence of EBV viremia and disease (4). In our study, myeloablation conditioning and use of ATG demonstrated a 3-times higher risk of developing EBV infection. ATG has been widely used to decrease the incidence of GVHD (31), however, because of T-cell depletion, ATG is also associated with relapse (32). The optimal dose of ATG depends on transplant characteristics, such as type of donor, and may range from 2.5 to 30 mg/kg (33). ATG has beneficial effects in preventing GVHD, although it delays immune reconstitution, promoting an increased risk of EBV reactivation, and potentially EBV-associated lymphoproliferative disease (34). Literature shows that higher doses of ATG seem to be related with PTLD development (35). Indeed, at our institution, no more than 10 mg/kg is used, and this may have contributed for the fact that no PTLD was observed in this cohort. When addressing the development of GVHD, we observed that patients who developed aGVHD, had 3-times increased association with EBV infection. Furthermore, we noticed that all patients with cGVHD, were positive for EBV at least once in the post-transplant follow-up. As described previously by Janeczko *et al*, GVHD is related to delayed immune reconstitution, favoring infections in the early period post-transplant. Moreover, viral infections are also associated with delayed immune response and appear to be linked to the degree of immunosuppression (36).

Review of EBV infection on different periods post-transplant revealed that unrelated donor, myeloablation and the use of ATG seem to be risk factors for EBV infection occurrence at D+60; while GVHD is connected to EBV infection at D+90; and ATG, unrelated donor and GVHD are related to EBV infection at day D+150. These results seem to corroborate the literature regarding the identification of high-risk markers for EBV infection. Furthermore, our study reports that EBV positivity at D+90 days and D+180 might be associated with increased mortality (P=0.095, 303.3 vs. 593.2 days and P=0.097, 367.0 vs. 679.5, respectively). We acknowledge that EBV infection is not, by itself, responsible for higher mortality rates, therefore further studies should be performed with more patients.

EBV infection is still a major concern in the subset of HSCT and these results showed that EBV routine monitoring is useful in high-risk patients during the first months after transplantation. In a previous study, the authors accessed the importance of EBV monitoring in patients submitted to aHSCT, regarding the development of PTLD (13). Although no patients of this study developed PTLD, it is still important performing EBV monitoring when several risk factors, are present. The implementation of guidelines and standardization of EBV monitorization in HSCT patients will contribute for the cost-effectiveness of this monitorization reducing unnecessary morbidity/mortality associated with EBV-infection.

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Availability of data and materials

The data obtained from the present study are part of the institution clinical records of patients and are not publicly available due to confidentiality but are available from the corresponding author on reasonable request.

Authors' contributions

JM-D, RM and HS designed the study. CP-V, LL, RB, FC and ACT collected and analyzed the clinical information of patients. IB and RM collaborated in viral detection and interpretation of results. JM-D and HS performed analysis of data, the draft of the manuscript and its final version. All authors were given the opportunity to revise the manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of Portuguese Oncology Institute of Porto and patients provided informed consent for enrollment in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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