"CAROL DAVILA" UNIVERSITY OF MEDICINE AND PHARMACY, BUCHAREST
DOCTORAL SCHOOL
FIELD OF MEDICINE

PhD THESIS SUMMARY

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INTERACTION BETWEEN MICROENVIRONMENT AND REED-STERNBERG CELLS: IMPACT ON TREATMENT RESPONSE AND IDENTIFICATION OF PROGNOSTIC FACTORS IN HODGKIN LYMPHOMA EVOLUTION

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INTRODUCTION

Hodgkin lymphoma is a hematologic malignancy derived from the B lymphocyte in the germinal center of the lymph nodes or from the post-germinal center B lymphocyte, and in rare cases, from the T lymphocyte. The specific tumor cells of this condition, known as Hodgkin and Reed-Sternberg (HRS) cells, are pathognomonic and are characterized by their large size, being either multinucleated or large mononucleated. However, these cells represent only a minority of the tumor mass, being surrounded by a cellular microenvironment, which plays a crucial role in the pathogenesis and progression of the disease [1].

Hodgkin lymphoma accounts for approximately 10% of all newly diagnosed lymphomas, with an incidence in Europe of 2.2 cases per 100,000 people per year and a mortality rate of 0.7 cases per 100,000 people per year. The disease is more commonly found in men than in women, presenting a bimodal incidence distribution, with a peak among young adults and those over 60 years old. The diagnosis of Hodgkin lymphoma should be made according to the WHO classification. Histologically, Hodgkin lymphoma is divided into two main subtypes: the classical variant and the nodular lymphocyte-predominant type [1].

The treatment of classical Hodgkin lymphoma has become one of the greatest successes of modern hematology, with a cure rate exceeding 90% for patients with early-stage disease and approaching the same value for patients with advanced disease when risk-adapted therapy is used [1]. However, the favorable prognosis of Hodgkin lymphoma entirely depends on access to a modern healthcare system with adequate medical care, which is unfortunately unavailable in many regions of the world [1].

This work provides a synthesis of the epidemiology of Hodgkin lymphoma and summarizes current knowledge on associated risk factors, personalized treatment, and both immediate and long-term treatment complications. The choice of this doctoral thesis is based on the importance of studying the complex interactions between the tumor microenvironment and Reed-Sternberg cells, which are central elements in the pathogenesis of Hodgkin lymphoma.

GENERAL PART

Overview of Hodgkin Lymphoma

According to the World Health Organization (WHO) classification, Hodgkin lymphoma is divided into two main variants: Classical Hodgkin Lymphoma (cHL) and Nodular Lymphocyte-Predominant Hodgkin Lymphoma (NLPHL) [1]. These two forms of Hodgkin lymphoma are distinguished by specific clinical, morphological, immunological, and molecular characteristics, which allow their differentiation through histopathological and immunohistochemical examination. Classical Hodgkin lymphoma, which accounts for approximately 95% of cases, is further subclassified into four histological subtypes: nodular sclerosis, mixed cellularity, lymphocyte-rich classical Hodgkin lymphoma, and lymphocyte-depleted classical Hodgkin lymphoma [2].

The incidence and mortality of Hodgkin lymphoma vary significantly by geography, being higher in industrialized countries compared to developing ones. Mortality is also higher in developing countries, reflecting limited access to modern therapies that can cure most patients with Hodgkin lymphoma [3].

Up to 40% of Hodgkin lymphomas are associated with the presence of the EBV virus. The viral genome can be found in Reed-Sternberg tumor cells. The presence of the intracellular viral genome indicates that the infection occurred before the tumor transformation. In patients with HIV-associated Hodgkin lymphoma, the viral genome has been detected in 80-100% of cases. Additionally, EBV infection has also been associated with other germinal center malignancies, such as Burkitt lymphoma and post-transplant lymphomas. The impact of EBV infection is controversial [4].

The diagnosis of Hodgkin Lymphoma (HL) is established through histopathological and immunohistochemical examination of sections from a paraffin block containing a fragment of the lymph node or tumor biopsy. The characteristic cell of classical Hodgkin lymphoma (cHL), known as the Reed-Sternberg (RS) cell, is large, with at least two nuclei and frequently eosinophilic nucleoli, while the mononuclear variant is known as the Hodgkin cell. Immunohistochemically, HRS cells are positive for CD30, and CD15 is co-expressed in most cases, although it may be absent in 20-25% of cases. CD45, BOB.1, and OCT-2 are usually negative, but PAX5/BSAP indicates B-cell origin, although expression is weaker

than in reactive B cells. CD20 is present in 30-40% of cases but is often limited to a subset of HRS cells [2].

Classical Hodgkin lymphoma (cHL) represents a complex model of interactions between Hodgkin-Reed Sternberg (HRS) tumor cells and an immune-active but ineffective tumor microenvironment (TME). Aberrant activation of HRS cells, caused by somatic mutations, triggers complex interactions with various immune cell subsets, influencing tumor progression and treatment resistance. The TME is essential in cHL, where HRS cells, although rare, are supported by an inflammatory microenvironment that constitutes most of the tumor mass. The altered genome of HRS cells activates signaling pathways that induce the secretion of growth factors, chemokines, and cytokines, thereby remodeling the TME and influencing the migration and activation of leukocytes [5], [6], [7].

Prognostic Factors in Hodgkin Lymphoma

Prognostic factors in Hodgkin lymphoma are essential for assessing the disease's progression and individualizing treatments to achieve the best therapeutic outcomes. **Environmental factors** include socioeconomic status, access to medical care, and the availability of appropriate therapy. Patients in developed countries benefit from rapid access to the necessary diagnostic investigations, the most effective and modern treatments, including bone marrow transplantation in case of relapse, and clinical trials for relapsed or refractory patients [8][9].

Factors related to the extent of the disease and tumor burden refer to the anatomical spread of the tumor, tumor biology, and the impact of the tumor on the host organism. Disease extent and tumor volume are crucial factors for stratifying treatment strategies in Hodgkin lymphoma.

Patient-related factors include age, which is the most important factor when analyzing overall survival and remains an independent factor for progression-free survival, and gender, with men having a poorer prognosis than women [9][10].

Constitutional symptoms, defined as unexplained fever >38°C, profuse night sweats, and weight loss >10% of body weight, are present in approximately 10-25% of patients with limited-stage disease and up to 70% of patients with advanced-stage disease. The presence of B symptoms is a risk factor, particularly in stage II disease with bulky masses, which is

not considered limited-stage disease. B symptoms are caused by the production of pro-inflammatory cytokines by Hodgkin tumor tissue, especially IL-1, TNF-alpha, and IL-6 [11][12][13].

Biochemical Characteristics of Hodgkin Lymphoma:

Anemia is commonly found in patients with Hodgkin lymphoma (HL), affecting approximately 40% of patients. It is usually a mild to moderate normocytic anemia with characteristics of simple chronic anemia. The prognostic threshold in the International Prognostic Score (IPS) is 10.5 g/dl [11]. Changes in the number and composition of white blood cells in peripheral blood are frequent at the diagnosis of HL and represent prognostic factors. Specific changes include an increase in the number of white blood cells due to an increase in neutrophils, a decrease in lymphocytes, the appearance of monocities, and eosinophilia. In the IPS, the prognostic threshold for leukocytes is 15,000/microL, and for lymphocytopenia, it is 600/microL or less than 8%. Low serum albumin levels are associated with a poorer prognosis in many hematologic malignancies, including HL. The IPS score defines albumin levels of 4.0 g/dl as the threshold. The erythrocyte sedimentation rate (ESR), despite its nonspecific nature, is one of the oldest prognostic factors for HL. It is still used to define early-stage HL as favorable or unfavorable. Elevated B2M levels may be due to increased release from immune system activation or proliferation or decreased renal clearance. B2M is a prognostic marker in many lymphomas, including HL [7], [8], [11], [12].

The cellular microenvironment is an essential component of tumor tissue, playing a crucial role in the pathogenesis of classical Hodgkin lymphoma (cHL) and nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL). It provides tumor cells with growth factors and suppresses the antitumor immune response. As tumor cells and reactive cells proliferate together, a complex interaction develops between them. Tumor cells actively manipulate and adapt the surrounding environment in their favor, developing mechanisms that allow them to evade the antitumor immune response [7].

Hodgkin Lymphoma Treatment

The treatment of Hodgkin lymphoma is determined through a detailed assessment of the disease stage, prognostic factors, patient’s age, and comorbidities. The chosen

therapeutic regimen follows the current recommendations from national and international guidelines, ensuring a standardized and optimized approach to managing this condition. For initial staging, a comprehensive history, physical examination, and complete imaging, including FDG PET and CT of the neck, chest, abdomen, and pelvis, are necessary [1].

First-Line Treatment in Early-Stage Disease

For patients with limited-stage disease without unfavorable prognostic factors, B symptoms, or bulky tumor masses, it is recommended to reduce the number of chemotherapy cycles (ABVD) to 2 cycles to limit long-term toxicity, followed by 30 Gy irradiation to the involved field (IFRT) [14], [15], [16], [17].

First-Line Treatment in Early-Unfavorable Disease

For patients with limited-stage disease with unfavorable prognostic factors, B symptoms, or bulky tumor masses, it is recommended the following approach:

First-Line Options:

- **ABVD for 2 cycles followed by restaging**, with the following therapeutic options depending on the PET-CT results:
    - **If PET-CT shows a Deauville score of 1-3:** continue with either 2 additional cycles of ABVD followed by radiotherapy or 4 cycles of AVD if radiotherapy is omitted [15], [18], [19].
    - **If PET-CT is positive (Deauville score 4-5):** switch to 2 cycles of escalated BEACOPP, followed by restaging and ISRT 30 Gy radiotherapy (Deauville score 1-3) [15], [18], [19].
    - **If, after the 2 cycles of escalated BEACOPP, the PET-CT examination shows a Deauville score of 4-5:** a re-biopsy is recommended. If the biopsy does not show the presence of tumor cells, continue with radiotherapy; however, if the biopsy confirms the presence of tumor cells, the disease should be considered refractory, and the patient will continue treatment according to the guidelines for refractory disease [15], [18], [19].

First-Line Treatment in Advanced-Stage Disease

After a decade of debates regarding first-line therapy, whether with six or eight cycles of ABVD or escalated BEACOPP, to optimally balance the chances of cure and the risk of complications, individualized therapeutic approaches guided by risk factors and early response to chemotherapy, directed by FDG PET CT, have become the therapeutic standard [20][21].

Another approach is the addition of brentuximab vedotin to AVD, with results showing a slight improvement in treatment response, but the regimen is accompanied by increased toxicity and costs**.** After decades of significant but slow progress in the treatment of advanced-stage HL, personalized therapeutic strategies have led to improved treatment options [22][23].

In the event of primary refractory disease, histological confirmation is recommended if progression occurs in another lymph node group. Biopsy is not mandatory if progression is confirmed by imaging at the initial site [21][23][24].

Salvage therapy for patients with relapsed/refractory disease must always be accompanied by consolidation with autologous peripheral stem cell transplantation**.** This approach represents the therapeutic standard in this situation. Salvage therapy must be personalized for each patient, considering the initial therapy, the cumulative toxicity of doses, patient comorbidities, and the need to obtain a peripheral stem cell harvest. Before starting chemotherapy, it is mandatory to assess cardiac and pulmonary function and conduct a fertility preservation consultation [1][25].

The objective of salvage therapy is to achieve a complete response to treatment confirmed by PET CT, which has a major impact on subsequent survival post-autologous peripheral stem cell transplantation**.** All second-line therapies are accompanied by hematologic toxicity. Infections and febrile neutropenia occur in 10-24% of patients. Nephrotoxicity, hepatotoxicity, mucositis, and digestive toxicity are observed in less than 10% of patients. Stem cell harvest is adequate with all therapeutic regimens [1][25].

Autologous peripheral stem cell transplantation is the standard treatment for patients with relapsed or refractory Hodgkin Lymphoma. Hematopoietic stem cells mobilized in the peripheral blood are the source of stem cells. The preferred conditioning

regimen depends on the center's experience, but the most used regimen is the BEAM type [26][27][28].

Hematopoietic Stem Cell Allotransplantation (allo-HCT) remains an important curative strategy for patients with classical Hodgkin Lymphoma (cHL) who relapse or progress after an autologous transplant (auto-HCT). The curative efficacy of allo-HCT is supported by data obtained from retrospective analyses and phase II clinical trials, although these have included a relatively small number of patients. Comparative analyses between patients who underwent allo-HCT and those who did not due to the lack of a donor highlight a significant benefit in terms of progression-free survival (PFS) and overall survival (OS) in allotransplanted patients. The current EBMT indications for allo-HCT in cHL are presented in Appendix 8 [29].

Survivors of Hodgkin Lymphoma face a significant risk of long-term cardiovascular and pulmonary complications, such as heart failure and pulmonary fibrosis, associated with the use of anthracyclines and thoracic radiotherapy [30], [31], [32], [33]. Primary prevention of cardiovascular risk can be achieved through basic measures of effective management of existing comorbidities, such as hypertension, cardiac dysfunction (systolic or diastolic), arrhythmias, and metabolic disorders [33], [34], [35], [36], [37], [38].

Additionally, these patients are at increased risk of developing secondary neoplasms, including acute myeloid leukemia and solid cancers, which underscores the necessity for continuous monitoring and effective management of comorbidities [39], [40], [41].

II. ORIGINAL PART

Working Hypothesis and General Objectives

This work provides a synthesis of the epidemiology of Hodgkin Lymphoma and summarizes current knowledge on associated risk factors, personalized treatment, and both immediate and long-term treatment complications. The choice of this doctoral thesis is based on the importance of studying the complex interactions between the tumor microenvironment and Reed-Sternberg cells, which are central elements in the pathogenesis of Hodgkin Lymphoma. A detailed understanding of how the microenvironment influences the response to therapy is essential for optimizing therapeutic regimens and improving patient prognosis. Reed-Sternberg cells play a pivotal role in interactions with various

cellular populations within the tumor microenvironment, including immune and stromal cells, thereby influencing not only tumor progression but also treatment sensitivity. By identifying prognostic markers associated with these interactions, the proposed thesis can contribute to a more precise stratification of patients based on individual risks and the development of personalized therapeutic approaches.

Although current treatment regimens for Hodgkin Lymphoma have demonstrated efficacy, they are often associated with significant toxicities. Thus, elucidating the mechanisms by which the tumor microenvironment modulates treatment response could facilitate the development of more selective therapeutic strategies with a reduced toxicity profile. This research direction has the potential not only to improve patient prognosis but also to advance knowledge in hematology, opening new perspectives on the therapeutic modulation of the tumor microenvironment to enhance the efficacy of existing treatments.

In the general part of the thesis, I briefly presented theoretical data representing the current state of knowledge on Hodgkin Lymphoma, emphasizing the particularities related to the clinical-biological characteristics of patients, prognostic factors, therapeutic options, and the outcomes of therapies adapted to accurate staging and risk factors. I introduced a subsection highlighting the importance of the tumor microenvironment in the subsequent evolution of the disease and the mechanisms of immune escape by tumor cells.

The work is essential for improving the clinical management of patients with classical Hodgkin Lymphoma, providing valuable data on treatment response and prognostic factors from a cohort treated in Romania. It also contributes to the personalization of therapies and the development of evidence-based clinical guidelines, with the potential to improve patient survival and quality of life.

The doctoral thesis consists of three studies that analyzed the data of patients diagnosed and treated at the Fundeni Hematology Clinic during the period 2017-2023, using the National Hodgkin Lymphoma Registry platform, a registry overseen by the Romanian Society of Hematology.

Study I: Single-Center Experience in a Retrospective Analysis of the National Hodgkin Lymphoma Registry in Romania

Hodgkin Lymphoma (HL) is a malignant hematologic condition of the lymph nodes and lymphatic system. There are two main subtypes: classical Hodgkin lymphoma (cHL) and nodular lymphocyte-predominant HL [42]. HL accounts for approximately 10% of all lymphomas. Globally, in 2020, this disease represented between 0.2% and 0.4% of the total number of newly diagnosed cancer cases [42]. Unfortunately, over the past decade, the existing literature has not provided sufficient information regarding disease progression, treatment response rates, survival rates, progression-free survival, treatment protocols used, and the existence of clinical trials for patients with Hodgkin Lymphoma in the Balkans. A review of the literature reveals a limited number of published articles from this geographical area.

Therefore, this study aims to comprehensively analyze the degree of treatment adherence among patients diagnosed with Hodgkin Lymphoma in Romania. Additionally, the study aims to evaluate demographic data, prognostic factors, classify these patients based on risk factors, monitor the use of international protocols in treatment selection, assess response rates, survival, and progression-free survival, as well as the use of modern methods in therapeutic decision-making. The study also seeks to compare this information with published data from the Balkans, thus providing an overview of the situation in this geographical region.

Materials and Methods

The study is a retrospective, observational cohort study. It included 182 patients over the age of 18 who were diagnosed with Hodgkin lymphoma between 2017 and 2023 at the Fundeni Clinical Institute.

Exclusion criteria: Patients who were not diagnosed at the Fundeni Clinical Institute, patients who died during the first admission or did not return to the clinic for continued treatment, and patients under 18 years of age.

The data were retrieved from the National Hodgkin Lymphoma Registry created by the Romanian Society of Hematology. The study was approved by the Local Ethics

Committee of the Fundeni Clinical Institute (No. 24768). All data from the registry were reviewed and updated until December 31, 2023.

Statistical Analysis

The study utilized data from the private national platform limfom.srh.org.ro, which is a specialized database used by doctors across the country to manage lymphoma cases. The platform uses a structured relational database management system, specifically MySQL, to store and organize clinical information. Data extraction and processing were carried out using the PHP programming language, without the use of additional frameworks. A specific set of selection criteria was used to create a representative sample of all cases recorded on the platform. The analysis involved the development of custom SQL queries to extract relevant data, filtering, and associating them. Subsequently, PHP scripts were used for data manipulation and aggregation.

Security measures on the platform were enhanced to provide additional protection for data extraction and use, ensuring comprehensive security. The collected data were fully anonymized to protect them against unauthorized access. The statistical analysis provides valuable insights into trends and patterns from a specific subset of data, offering a perspective focused on cases that meet the established inclusion criteria.

The results of the analysis are briefly summarized in tables and statistical charts using HTML tools and MS Excel, facilitating clear interpretation and presentation of the study's conclusions. The data processing methodology was meticulously developed to ensure the accuracy and relevance of the results, closely aligning with the specific research objectives.

To estimate progression-free survival (PFS), disease-free survival (DFS), and overall survival, the Kaplan-Meier method implemented in the lifelines library in Python 3 was used, while the matplotlib library was used for visualizing the curves. Survival probabilities were extracted at specific time points (6, 12, and 36 months), and log-rank tests were performed to statistically compare survival distributions between stages. In this analysis, Progression-Free Survival (PFS) was defined as the time until the recording of progressive disease (PD) or death. Patients who achieved complete response (CR), partial response (PR), or stable disease (SD) were not counted as having progressed; Disease-Free Survival (DFS) was

defined as the time after CR without subsequent relapse of the disease or death. If the first-line treatment did not produce a CR, a DFS of 0 was assigned.

Results

Of the total patients studied, 75.27% were aged ≤ 45 years, 19.78% were between 45 and 65 years old, and 3.85% were older than 65 years. The data indicate that the age distribution of Hodgkin lymphoma patients in Romania exhibits a bimodal age distribution, like global trends, with incidence peaks among young adults and older adults. This demographic pattern highlights the need for targeted public health initiatives aimed at these age groups to improve early detection and intervention strategies (7).

Among the patient population studied, 47.25% were men and 52.75% were women, with a male-to-female ratio of 0.90:1. There is a difference compared to the distribution presented in the literature, where males are typically more prevalent. This gender disparity may be related to increased anxiety among women in Romania regarding the diagnosis and their preference to seek treatment at a university medical center (87).

Registry data indicate that patients from urban areas generally have better access to medical care, which is why most patients in the study come from urban areas (61%). This discrepancy between urban (61%) and rural (39%) areas in terms of access to healthcare leads to disparities in treatment outcomes, with rural patients facing delayed diagnoses, reduced adherence and compliance to treatment, and suboptimal therapeutic regimens.

The data show that 82 patients (45.06%) were diagnosed with limited-stage disease (IA, IB, IIA, IIB), while 54.94% were diagnosed with advanced-stage disease (IIIA, IIIB, IVA, IVB). There is a significant difference compared to the literature, where most patients are diagnosed in stages I and II. This difference is also reflected in the presence of B symptoms; in the present study, a significant percentage of patients (62.64%) presented with B symptoms at diagnosis, compared to what is reported in the literature [43].

It was observed that 60.99% of the patients included in the study presented with the nodular sclerosis histological subtype, 28.57% presented with the mixed cellularity histological subtype, 3.85% presented with the lymphocyte-rich histological subtype, and 2.20% with the nodular lymphocyte-predominant histological subtype. The immunohistochemical phenotype of HRS cells in NSCHL is the classic one, but association

with the Epstein-Barr virus (EBV) is less common compared to other cHL subtypes, especially mixed cellularity (MCCHL) [1], [42], [44]. This distribution is like that described in the literature [43]. It is also worth noting that a low percentage of patients (3.85%) in the studied group had an unclassifiable histological subtype. The histological subtype could not be clearly determined due to the small amount of tumor tissue (needle biopsy) or the use of bone marrow biopsy for diagnosis.

The International Prognostic Score (IPS) is the most used tool for determining prognosis, published by the German Study Group and has been used for more than 25 years. The IPS is important because it provides a method to assess risk and anticipate disease progression in patients with advanced-stage Hodgkin lymphoma. In the present study, among patients diagnosed with advanced-stage disease, it was observed that 66.67% had an IPS score of 1.

Regarding Bulky tumor mass, a larger number of patients were observed to not have a Bulky tumor mass at diagnosis, accounting for 84.62%. However, 15.38% of patients did present with a Bulky tumor mass, which is almost double the value reported in the study published in the Balkan Medical Journal, where 9% of patients were diagnosed with this type of tumor [43]. From this significant difference, one can deduce an issue with the accessibility of healthcare services for patients in Romania, which results in patients presenting to the doctor at a more advanced stage of the disease.

B symptoms were selected according to NCCN guidelines and are represented by constitutional symptoms, defined as unexplained fever >38°C, profuse night sweats, and weight loss >10%. In this study, it was observed that most patients who presented with B symptoms at diagnosis were diagnosed in stage IV (66.67%) and stage III (65.22%) of the disease. This highlights the fact that the percentage of patients with favorable limited-stage Hodgkin lymphoma is low, which could explain the lower cure rate compared to the cure rate reported in the literature. However, the presence of B symptoms can be subjective, depending on the season, the patient's psychological profile, and hormonal status.

Biochemical Aspects of Hodgkin Lymphoma:

Low serum albumin levels are associated with a poorer prognosis in many hematological malignancies, including Hodgkin lymphoma (HL). The International

Prognostic Score (IPS) defines albumin levels of 4.0 g/dl as the threshold. Most of the patients at diagnosis had normal albumin levels (90.7%). It was observed that most patients with low albumin levels were diagnosed in stage III and IV of the disease (6.60%). Regarding hypoalbuminemia, there is a noticeable difference between patients diagnosed with limited stages of the disease, who present a lower percentage of hypoalbuminemia, compared to those diagnosed in advanced stages of the disease. This difference is likely due to liver involvement in the disease, which decreases albumin synthesis.

Anemia is commonly found in patients with Hodgkin lymphoma (HL), occurring in approximately 40% of patients according to data from the literature. It is usually a simple, chronic anemia, mild to moderate in severity, with characteristics of simple chronic anemia. The threshold for prognosis in the International Prognostic Score (IPS) is 10.5 g/dl [1]. Among the total number of patients studied, there was a predominance of anemia among those diagnosed in stage III and IV of the disease (22.50%) compared to those diagnosed in stage I or II of the disease. From these results, it can be deduced that anemia is an indicator of the presence of advanced stages of the disease. As described in the literature, anemia is even considered a negative prognostic factor [43].

Changes in the number and composition of white blood cells in peripheral blood are common at the diagnosis of Hodgkin lymphoma (HL) and represent prognostic factors. Specific changes include an increase in the number of neutrophils, a decrease in the number of lymphocytes, and the occurrence of monocities and eosinophilia [1]. Leukocytosis (white blood cells > 15,000/mm^3) was recorded in just over one-third of all patients, with the highest prevalence among those diagnosed in stage IIIB and IVB of the disease. However, there was no statistically significant difference in the incidence of leukopenia between patients diagnosed in early stages compared to those diagnosed in advanced stages of the disease ($p < 0.05$). The percentage of those who presented with lymphopenia was 33.50%, with the highest prevalence among those diagnosed in stage IIIB and IVB of the disease (19.80%), a characteristic like the distribution of hypoalbuminemia and anemia among patients.

In lymphoma pathology, the presence of elevated C-reactive protein (CRP) levels reflects an increase in inflammatory cytokines, particularly elevated IL-6 levels (which is associated with the development of malignant processes). In this study, as also reported in the literature, elevated CRP levels were found in advanced stages of the disease (IIIB, IVB)

at 28.60%, compared to early stages IA, IIA (5.50%), but without reaching statistical significance ($p > 0.05$).

LDH (lactate dehydrogenase) levels are an important prognostic factor in non-Hodgkin lymphoma, but they have been less studied in Hodgkin lymphoma, although it has been shown that LDH could have considerable value as an independent factor, potentially more significant than other parameters such as age. Moreover, it is an objective and easily accessible parameter. In our study, elevated LDH levels were associated with advanced stages of the disease IIIB, IVB (24.70%) compared to early stages IA, IIA (2.70%), but without reaching statistical significance ($p < 0.05$).

Treatment in Hodgkin Lymphoma

There has been considerable debate regarding the optimal first-line therapy, whether to administer six or eight cycles of ABVD or escalated BEACOPP, to balance the chances of cure with the risk of complications. Currently, individualized therapeutic approaches, guided by risk factors and early response to chemotherapy as determined by FDG PET-CT, have become the standard treatment in many developed countries [45]. In the present study, it was observed that both ABVD and BEACOPPesc therapies were most frequently used in stage II of the disease, with 49.60% and 56.30% of the total patients, respectively, receiving these treatments. The results align with the information presented in the literature [43].

Stages IA, IIA with Favorable Prognosis: This stage of the disease includes patients with involvement on one side of the diaphragm only and without negative prognostic factors such as Bulky tumor mass, B symptoms, or many affected lymph node groups. In the present study, all patients were administered chemotherapy. In the early stages of the disease, ABVD therapy was administered in all cases as the first-line treatment; it was observed that 26 out of 34 patients (76.5%) achieved a complete response at the interim evaluation. Four patients who did not initially achieve a complete response required treatment escalation to BEACOP (1 patient, 2.94%) or BEACOPPesc (3 patients, 8.82%), resulting in a complete response. In conclusion, 29 patients, representing 85.3% of the total, achieved a complete response. Although radiotherapy is currently considered an obligatory part of the therapeutic regimen for limited stages, it was not administered to all patients in this study, most likely because some patients opted out of receiving it and preferred to undergo two additional cycles of chemotherapy instead. Among the patients who received radiotherapy as part of the initial

regimen, 93% had a complete response at the end of treatment. Most radiotherapy specialists avoid using radiotherapy on the primary affected area and prefer to administer it to the residual tumor.

Stage IA, IIA with Bulky Tumor, IB, IIB: These stages represent limited disease but with an unfavorable prognosis due to the presence of a Bulky tumor mass or general disease symptoms, stages IB, IIB. The therapeutic approach is more aggressive than that used in stages IA, IIA. Typically, the ABVD regimen is initiated for two cycles, followed by imaging reassessment. Based on the PET-CT results, the future therapeutic approach is decided. All patients in these stages (48, 26.4% of the total studied) were administered chemotherapy. In these stages, ABVD therapy was administered to 37 patients (77.1%) as the first-line treatment; it was observed that 25 out of 48 patients (52.1%) achieved and maintained a complete response at the interim evaluation. Three of these patients did not initially achieve a complete response and required treatment escalation to BEACOPP (1 patient) or BEACOPPesc (2 patients), resulting in a complete response in both cases. In conclusion, 28 patients who were administered ABVD therapy achieved a complete response.

Stage III and IV Disease: These stages include Hodgkin lymphoma patients with involvement of lymph node regions on both sides of the diaphragm and/or with disease spread to one or more extra nodal organs. In these stages, the standard is the administration of 6 cycles of chemotherapy, regardless of the selected therapeutic regimen. The standard and most used regimen are ABVD, but in cases with unfavorable prognostic factors, more aggressive treatment is required, such as up to 6 cycles of BEACOPPesc. In this study, all patients in this stage of the disease (100) initially received chemotherapy. Most of the patients were treated with the ABVD regimen, with a complete response rate of 52.54% at the interim evaluation. Seven patients did not initially achieve a complete response and required treatment escalation to BEACOPPesc (6 patients) or IGEV (1 patient), resulting in a complete response for these patients. In conclusion, a total of 62.7% of stage III or IV Hodgkin lymphoma patients treated with ABVD therapy achieved a complete response. BEACOPPesc treatment was used in 6% of cases as the first line in these stages of the disease, with a final complete response rate of 33.33%. Additionally, 17 patients received AVD plus Brentuximab Vedotin as first-line treatment, achieving the best response rate (70.6%). Radiotherapy was used less frequently for stage III and IV Hodgkin lymphoma

patients due to a high rate of complete response after chemotherapy, with no active residual disease confirmed by PET-CT examination with a Deauville score of ≤ 3.

Relapsed/refractory Disease:

Refractory or relapsed Hodgkin lymphoma (RRHL) presents significant clinical challenges, often requiring more aggressive and multifaceted treatment strategies. Primary refractory disease is characterized by the lack of response to initial therapy, while relapse refers to the reappearance of the disease after an initial period of remission [46], [47], [48]. Among the cases followed in this study, 38.46% (n=70) required continuation of treatment due to a suboptimal response to initial treatment or relapse after first-line therapy. In stages IA and IIA, the most used therapy was ABVD, accounting for 70.7% of cases. For advanced stages, BEACOPPesc was the predominant second-line therapy, representing 45.5%. ICE/IGEV/DHAP was also used in advanced stages as second-line therapy in 41.7% of cases. Among the patients who required second-line treatment, 17.1% were treated with autologous transplantation.

To estimate progression-free survival (PFS), disease-free survival (DFS), and overall survival, the Kaplan-Meier method implemented in the lifelines library in Python 3 was used, while the matplotlib library was used for visualizing the curves. Survival probabilities were extracted at specific time points (6, 12, and 36 months), and log-rank tests were performed to statistically compare survival distributions between stages. In this analysis, Progression-Free Survival (PFS) was defined as the time until the recording of progressive disease (PD) or death. Patients who achieved complete response (CR), partial response (PR), or stable disease (SD) were not counted as having progressed; Disease-Free Survival (DFS) was defined as the time after CR without subsequent relapse of the disease or death. If the first-line treatment did not result in a CR, a DFS of 0 was assigned.

As observed in the specialized literature, in this study, patients diagnosed at early stages had a longer progression-free survival compared to those diagnosed at advanced stages of the disease. There was a statistically significant difference (p=0.0431) in progression-free survival between stage II and stage IV of the disease.

The results of our study are similar to those of the US SEER study in terms of predominant histological type, average age at diagnosis, male-to-female ratio, and the

prevalence of advanced-stage disease at diagnosis. A significant difference between our study and the US SEER study is the small number of patients diagnosed at stage I. This discrepancy can largely be attributed to the absence of PET-CT examinations at the time of diagnosis in our study, where only CT scans were performed. This limitation led to a tendency for over staging due to concerns about possible underestimation, which could lead to insufficient treatment and a higher rate of therapeutic failure, underscoring the crucial role of advanced imaging in accurate staging. Advanced-stage diagnosis is like that recorded in Balkan registries, a situation mainly attributed to patients' reluctance to undergo additional medical examinations, as well as the lack of medical education. Patients avoid seeing a doctor, self-medicate, and delay seeking a specialist. Another issue is the lack of involvement of the family doctor in directing the patient, often prescribing antibiotics, and anti-inflammatory therapy [49], [50][51].

Conclusions

- The average age of the diagnosed patients was 35 years, with 47.25% being men and 52.75% women, resulting in a male-to-female ratio of 0.90:1. Most of the patients (75.27%) were 45 years old or younger, consistent with the global trend of Hodgkin lymphoma (HL) predominantly affecting younger individuals.
- The most frequently observed histological subtype was nodular sclerosis, accounting for 60.99% of cases, followed by mixed cellularity at 28.57%.
- At the time of diagnosis, stage II was the most common (43.41%), followed by stage IV (29.67%), indicating that a significant proportion of patients were diagnosed at an advanced stage. Limited-stage disease (stages I and II) was diagnosed in 45.06% of patients, while advanced-stage disease (stages III and IV) was present in 54.94% of cases.
- In the initial stage, all cases were treated with ABVD therapy as the first-line treatment, and it was found that 26 out of 34 patients (76.5%) achieved a complete response at the interim evaluation. Of the remaining patients, 4 did not initially achieve a complete response and required intensified treatment, which resulted in a complete response for all. In summary, 29 patients, representing 85.3% of the total, achieved an overall complete response.

- In stages IA, IIA with Bulky disease, IB, and IIB, ABVD therapy was administered to 37 patients (77.1%) as first-line treatment; it was found that 25 out of 48 patients (52.1%) achieved a complete response at the interim evaluation and maintained it. Four patients did not initially achieve a complete response and required intensified treatment, which resulted in a complete response for all (82.4%). In conclusion, 28 patients treated with ABVD therapy achieved a complete response.
- In the cohort of patients with stage IV Hodgkin lymphoma, 45.9% (17 individuals) underwent first-line therapy with the AVD regimen (Adriamycin, Vinblastine, and Dacarbazine) plus Brentuximab Vedotin. At the interim evaluation, it was found that 76.5% of the patients achieved a complete response, indicating an improved overall response rate.
- In stages III and IV, the overall response rate to ABVD was 50.85%, significantly lower than the response rate to AVD-Brentuximab, which was 76.5%.
- One of the major differences between our study and other studies, such as the US SEER study, is the small number of patients diagnosed at stage I.
- This discrepancy can be largely attributed to the absence of PET-CT scans at the time of diagnosis in our study, where only CT scans were performed. This limitation led to a tendency for overstaging due to concerns about possible underestimation, underscoring the crucial role of advanced imaging in accurate staging.

Study II: Impact of Exposure to Brentuximab Vedotin Therapy on T Cell Populations and Clinical Outcomes in Patients with Advanced Hodgkin Lymphoma

Hodgkin lymphoma (HL) is a hematologic malignancy primarily located in the lymph nodes and less commonly in extra nodal 2sites. HL is classified into classical Hodgkin lymphoma (cHL), which accounts for approximately 95% of cases, and a smaller subgroup, nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL), which accounts for about 5% of cases. Histologically, cHL is characterized by a small number of mononuclear Hodgkin cells and multinucleated Reed-Sternberg (HRS) cells, whereas lymphocyte-predominant (LP) cells are more frequent, being a characteristic of NLPHL [53], [54].

The tumor microenvironment present in classical Hodgkin lymphoma (cHL) is actively shaped through the recruitment of cells driven by a complex profile of growth factors, chemokines, and cytokines released by the Hodgkin Reed-Sternberg (HRS) cells. The mechanisms developed by HRS cells create an immunosuppressed environment that is conducive to tumor development while also avoiding detection by the immune system [55]. The tumor microenvironment in cHL primarily comprises infiltrates of T cells, including CD8+ cytotoxic T lymphocytes (CTLs), CD4+ helper T cells (Th), and CD4+ regulatory T cells (Tregs) [7].

Emerging therapies, including immune checkpoint blockade and targeted treatments, can influence Treg activity and may lead to the occurrence of adverse reactions. Therefore, it is crucial to find a balance between enhancing antitumor immunity and minimizing adverse reactions to improve treatment outcomes in the future. The efficacy of Brentuximab Vedotin (BV) was established through a phase III randomized clinical trial that studied patients with newly diagnosed Hodgkin lymphoma as well as those with relapsed or refractory disease [56].

Study Objectives

To explore the potential relationship between Brentuximab Vedotin (BV) and Tregs in Hodgkin lymphoma, our study aimed to examine the immune microenvironment in patients with stage IV Hodgkin lymphoma before and after treatment with BV. Specifically, we focused on analyzing biomarkers expressed in the tumor microenvironment, which have been shown to have prognostic significance in recent studies. The biomarkers evaluated in peripheral blood included markers of regulatory T cells (Tregs) (CD4+CD25+CD127low/-, FOXP3+), the B cell marker (CD19+), and the markers for CD3, CD4, and CD8 T cells, which identify non-specific T cells, T-helper cells, and cytotoxic T cells, respectively, as well as NK cells.

Materials and Methods

The study is a prospective cohort study. Fifteen patients diagnosed with stage IV classical Hodgkin lymphoma (advanced stage, highlighting the aggressive and disseminated nature of the disease at the time of diagnosis) were included in the study. The study adhered to the ethical principles outlined in the Declaration of Helsinki and received approval from

the Ethics Committee of the Fundeni Clinical Institute (15417/29.03.2024). Written informed consent was obtained from all patients in accordance with the requirements of the General Data Protection Regulation (GDPR). All patients received first-line treatment with doxorubicin, vinblastine, dacarbazine (AVD) plus Brentuximab Vedotin, in accordance with International Guidelines.

Peripheral blood samples were collected in the Hematology Department of the Fundeni Clinical Institute, Bucharest, Romania, before and after treatment (with a follow-up period ranging from 6 to 20 months). For two patients, treatment had to be changed due to partial response and disease progression.

Used techniques:

- **Isolation of Peripheral Blood Mononuclear Cells (PBMCs):** PBMCs were isolated by density gradient separation using Ficoll-Paque PLUS (GE Healthcare Biosciences, USA) with a density of 1.077 g/ml. The number of PBMCs was manually estimated using trypan blue and a hemocytometer.
- **Cell Viability Analysis:** A Stem reagent kit (Beckman Coulter, USA) was used, which included a murine monoclonal antibody (CD45-FITC Isoclonic Control-PE) and a viability dye (7-AAD) to identify and count viable leukocytes (CD45+ populations) from fresh or thawed PBMCs through flow cytometry.
- **Analysis of Lymphocyte Subsets:** To calculate the absolute number of lymphocyte subsets, 50 µL of well-mixed anticoagulated whole blood was added to BD Trucount™ tubes along with 20 µL of BD Multitest™ 6-color TBNK reagent (Fluorochrome-labeled antibodies: CD3 FITC, CD16 PE + CD56 PE, CD45 PerCP-Cy5.5, CD4 PE-Cy7, CD19 APC, CD8 APC-Cy7). The results were expressed as the percentage of lymphocytes that are total T cells (CD3+), CD3+CD4+, CD3+CD8+, B lymphocytes (CD19+), and NK lymphocytes (CD3–CD16+ and/or CD56+).
- **Identification of Regulatory T Cells CD45+ CD4+ CD25+ CD127dim/neg FoxP3+ by Flow Cytometry**

Statistical Analysis

Data was collected in Excel and analyzed using Python 3. The Pandas library was used for data structuring, column statistics, and selection of comparison parameters, while Matplotlib was used for visualization. The statistical significance of the concentration of each cell type between time periods was tested using the two-tailed Student's t-test provided by the SciPy library. P-values less than 0.05 were considered statistically significant. Since patients attended follow-up visits at different times after the initiation of treatment, the values measured at 5-7 months, 8-10 months, and 11-20 months were grouped and used for comparison of cell numbers.

Results

At 8-10 months post-treatment, a significant decrease in the number of CD3+ T lymphocytes was observed, with an average reduction from 80.4% to 74.45% (statistically significant, $p = 0.040$); however, this was followed by a recovery to levels like those before treatment within the 11–20-month interval. The dynamic changes in lymphocyte populations, in this case, the decrease followed by the recovery of CD3+ T lymphocyte levels, align with the immune reconstitution patterns observed in other studies conducted post-chemotherapy.

The populations of Treg cells showed a constant increase (over 3 times the initial values) after treatment administration. From a median value of 0.38% of total lymphocytes before treatment to 1.04% in the 8–10-month interval, and subsequently to 1.43% in the 11–20-month interval (statistically significant, $p = 0.033$). These dynamic changes in lymphocyte populations, specifically the significant increase in Treg cell populations, are consistent with the literature, indicating that chemotherapy may lead to an expansion of regulatory T cells, with the potential to influence immune surveillance and relapse rates.

For CD19 cells, although the increase in the number of these cells (B CD19+) was not statistically significant, the average values doubled from 4.3% before treatment to 9.5% in the 11–20-month post-treatment interval, without reaching statistical significance ($p = 0.165$).

NK cells, identified as CD3-CD56+CD16+ lymphocytes, recorded an increase in the first-year post-treatment, with an average value of 19.9% in the 8–10-month interval

compared to an average value of 11.3% observed before therapy administration; however, this increase was not statistically significant (p = 0.116).

The present research confirmed that T lymphocytes represent the most abundant component of the microenvironment in classical Hodgkin lymphoma (cHL). Previous studies have demonstrated that in vitro experiments show a selective overexpression of the protein galectin-1 (Gal1), an immunoregulatory protein that binds to glycans, in Hodgkin Reed-Sternberg cells (HRSC) and Hodgkin cells (HC) through an AP1 (activator protein 1)-dependent enhancer, as well as TGF-β (transforming growth factor beta-1). These proteins contribute to the secretion of Th-2 cytokines and the proliferation of CD4+/CD25high/FOXP3+ regulatory T cells (T-reg cells).

The role of B cells in classical Hodgkin lymphoma (cHL) remains considerably ambiguous. Non-malignant B cells are abundant in nodular lymphocyte-predominant Hodgkin lymphoma, where treatment with anti-CD20 monoclonal antibodies is effective. Targeting B cells by administering Rituximab, both as monotherapy and in combination with standard treatment regimens, has shown therapeutic potential.

However, the existing microenvironment in classical Hodgkin lymphoma (cHL) remains largely unexplored, and to elucidate its complex structural and functional nature, the use of the techniques mentioned before is necessary.

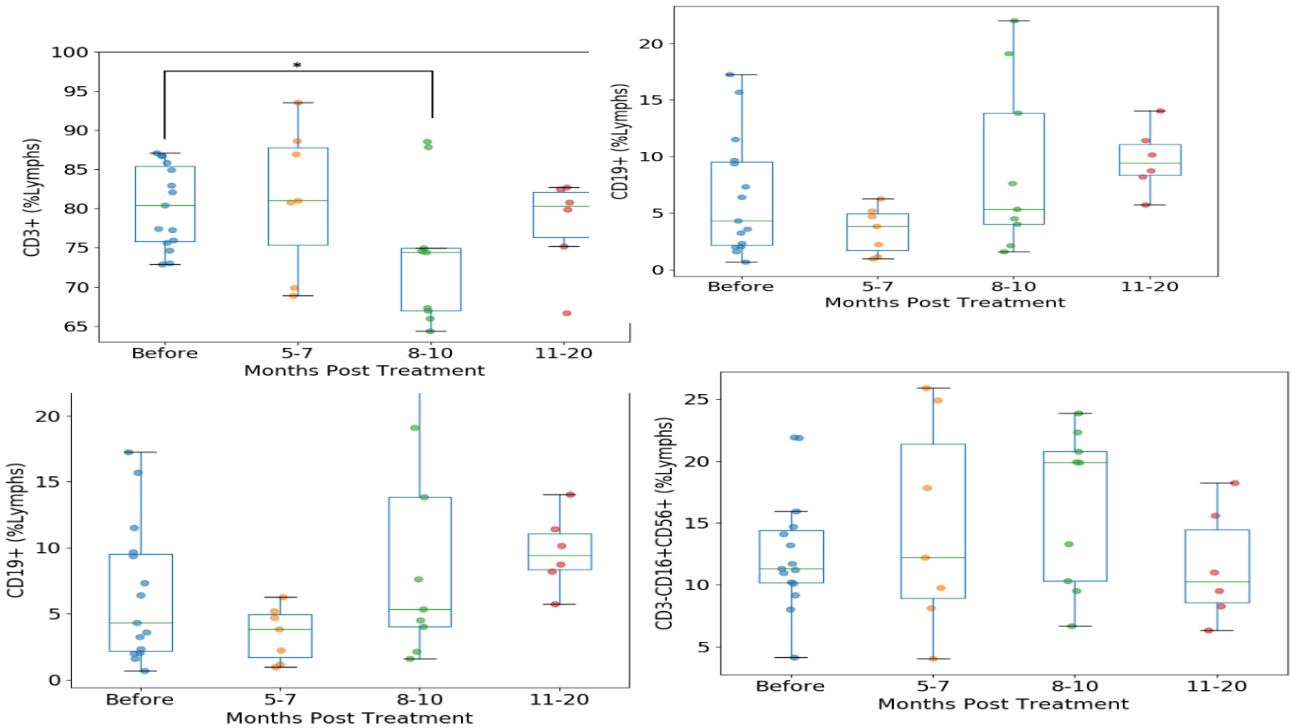

Figure 1. The Evolution of Tumor Microenvironment Cells Over Time

Personal Contributions

This work provides a detailed characterization of the patient population diagnosed with Hodgkin Lymphoma between 2017-2023 at the Fundeni Clinical Institute, the largest hospital in Bucharest, Romania. It represents the largest study conducted to date in Romania, including demographic data, disease stage at diagnosis, clinical, biological, and imaging data, as well as an analysis of prognostic factors, therapeutic options used, and evaluation of treatment response. This extensive database is remarkable because it allows for a comprehensive assessment of the typical patient profile in a geographical region with limited published data, providing relevant information on diagnostic and treatment trends, as well as on the implementation of diagnostic and treatment guidelines.

The work analyzes the effectiveness of existing therapeutic regimens, such as ABVD and BEACOPP, as well as the recent implementation of the AVD + Brentuximab Vedotin protocol in patients with stage IV disease, who have the worst prognosis among Hodgkin Lymphoma patients, as well as the patients' response to salvage therapy and access to autologous stem cell transplantation. This aspect is particularly important because it allows for the evaluation of therapeutic response based on disease stage and individual patient characteristics, contributing to personalized therapeutic approaches.

The originality of this work lies in its contribution to the National Hodgkin Lymphoma Registry. The inclusion of patients in the National Hodgkin Lymphoma Registry is an innovative aspect that facilitates the comparison of national data with literature data and helps identify diagnostic and treatment issues, evaluating access to treatment in different areas of the country. This work supports future research efforts and contributes to the improvement of public health policies.

In Chapter 2 of the thesis, I analyzed the impact of Brentuximab Vedotin therapy on lymphocyte populations in patients with stage IV Hodgkin Lymphoma at diagnosis, and then in dynamics, highlighting the significant increase in Treg cell populations. This finding is

consistent with the literature, indicating that chemotherapy may lead to the expansion of regulatory T cells, with the potential to influence immune surveillance and relapse rates.

NK cells also showed an increase in the first year post-therapy, which, although not statistically significant, is described in the literature as being correlated with a favorable response to treatment. The conclusions underscore the complexity of the tumor microenvironment and the need for further studies to fully understand the role of different cellular populations in this disease.

In the final chapter of the original part, I chose to present a series of cases of newly diagnosed stage IV Hodgkin Lymphoma patients who also had associated autoimmune pathology, a pathology influenced by Brentuximab Vedotin therapy. This represents the first work to describe the impact of Brentuximab Vedotin therapy in patients who also have autoimmune pathology.

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