Hodgkin Lymphoma – an Interplay Between Tumour Cell and Microenvironment

INGRID GLIMELIUS
Dissertation presented at Uppsala University to be publicly examined in Auditorium Minus Gustavianum, Akademigatan 3, Uppsala, Saturday, May 16, 2009 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract


Hodgkin lymphoma (HL) is a malignant disorder characterised by few tumour cells surrounded by a massive infiltrate of inflammatory cells, fibrosis, and microvessels. Therefore, it is a good model in which to study the interplay between tumour cells and the microenvironment.

In a population-based series, stage IIB had poor prognosis, equivalent to the most advanced stage (stage IV). The most prominent negative prognostic factor was tumour bulk in the mediastinum (often large fibrotic tumours).

The tumour cells expressed interleukin-9 (IL-9) in their cytoplasm in half of the cases. These cases had an over representation of nodular sclerosis histology (characterised by fibrotic bands) and infiltration of eosinophils and mast cells in the tumours. Despite this, IL-9 expression was not a negative prognostic factor.

A role of inflammatory cells is to contribute to angiogenesis. Yet, a correlation between high microvessel count and high mast cell number in HL tumours was not identified, in contrast to other lymphomas. However, a correlation to poor prognosis was seen for cases with high microvessel count.

Eosinophils contain eosinophil cationic protein (ECP). ECP was cytotoxic to cells from two HL cell lines of B-cell origin and one HL line of T-cell origin. At high concentrations, the cytotoxic effect was not as pronounced for the line of T-cell origin. If the in vitro cell lines are representative of HL in vivo, eosinophils may have different roles in different HL tumours.

In addition to the effect from tumour cells, host-related factors contribute to the inflammatory infiltrate in HL. A history of asthma and hives, and carrying the ECP434GG genotype were associated with elevated numbers of eosinophils, whereas, history of tobacco smoking was associated with lower numbers.

HL is a complex tumour consisting of recruited and subverted normal cells, fibrosis and angiogenesis: these constitute the microenvironment, which likely supports tumour cell growth, and differs between patients.

Keywords: Hodgkin Lymphoma, angiogenesis, bulky disease, eosinophil granulocyte, eosinophil cationic protein (ECP), mast cell, interleukin-9, microenvironment

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


IV Glimelius, I*., Eriksson, J.*, Fischer, M., Molin, D., Amini, RM., Venge, P. Enblad, G. *Contributed equally The effect of Eosinophil cationic protein on Hodgkin lymphoma tumour cell lines, manuscript*


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<tr>
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<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADCC</td>
<td>Antigen Dependent Cellular Cytotoxicity</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CHL</td>
<td>Classical Hodgkin Lymphoma</td>
</tr>
<tr>
<td>CS</td>
<td>Clinical Stage</td>
</tr>
<tr>
<td>CT</td>
<td>Chemotherapy</td>
</tr>
<tr>
<td>DFS</td>
<td>Disease Free Survival</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>ECP</td>
<td>Eosinophil Cationic Protein</td>
</tr>
<tr>
<td>EFRT</td>
<td>Extended Field Radiotherapy</td>
</tr>
<tr>
<td>EORTC</td>
<td>European Organisation for Research and Treatment of Cancer</td>
</tr>
<tr>
<td>ESR</td>
<td>Erythrocyte Sedimentation Rate</td>
</tr>
<tr>
<td>FFTF</td>
<td>Freedom from Treatment Failure</td>
</tr>
<tr>
<td>GHSG</td>
<td>German Hodgkin Study Group</td>
</tr>
<tr>
<td>HL</td>
<td>Hodgkin Lymphoma</td>
</tr>
<tr>
<td>HRS</td>
<td>Hodgkin and Reed-Sternberg cell</td>
</tr>
<tr>
<td>HLS</td>
<td>Hodgkin Lymphoma-specific survival</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IFRT</td>
<td>Involved Field Radiotherapy</td>
</tr>
<tr>
<td>INRT</td>
<td>Involved Node Radiotherapy</td>
</tr>
<tr>
<td>IPS</td>
<td>International Prognostic Score</td>
</tr>
<tr>
<td>LDHL</td>
<td>Lymphocyte Depleted Hodgkin Lymphoma</td>
</tr>
<tr>
<td>LRCHL</td>
<td>Lymphocyte Rich Classical Hodgkin Lymphoma</td>
</tr>
<tr>
<td>MCHL</td>
<td>Mixed Cellularity Hodgkin Lymphoma</td>
</tr>
<tr>
<td>MVC/MVD</td>
<td>Microvessel Count/Microvessel Density</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear Factor Kappa-B</td>
</tr>
<tr>
<td>NLPHL</td>
<td>Nodular Lymphocyte Predominant Hodgkin Lymphoma</td>
</tr>
<tr>
<td>NSHL</td>
<td>Nodular Sclerosis Hodgkin Lymphoma</td>
</tr>
<tr>
<td>OS</td>
<td>Overall Survival</td>
</tr>
<tr>
<td>PET</td>
<td>FDG-Positron Emission Tomography</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression Free Survival</td>
</tr>
<tr>
<td>PS</td>
<td>Pathological Stage</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RT</td>
<td>Radiotherapy</td>
</tr>
<tr>
<td>SCALE</td>
<td>SCAndinavian Lymphoma Etiology study</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue Microarray</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
</tbody>
</table>
Introduction

Hodgkin Lymphoma

Hodgkin lymphoma (HL) is a malignant disorder, evolving from lymphoid tissue [1]. In Sweden, approximately 160 people per year are diagnosed with HL and among them many are young adults [2]. The short time prognosis, especially for early stages, is excellent. However, patients with relapsed or primary progressive disease have poor prognosis and young patients still die from the disease [3]. Many patients also suffer from severe secondary complications from the intensive chemo- and/or radiotherapy used for cure [4-7]. Therefore, a continued search for new prognostic markers is important and will help for further optimisation of treatment. The goal is to provide enough treatment so the patients are cured, without giving too much so they suffer unnecessarily from complications. The desire is to also develop new less toxic drugs, that do not cause drug resistance of the tumour cells and that act synergistically with conventional treatment.

Most patients with untreated HL, including those with limited disease, have a defect in cell-mediated immunity which makes them more susceptible to infections. Whether the observed impaired immunity is the result of HL or constitutes a predisposition for HL is unclear [8].

In several ways HL can be seen as a model disease for other malignancies:

- Firstly, an HL tumour which is characterised by a few malignant cells, Hodgkin and Reed-Sternberg (HRS) cells, surrounded by a massive infiltrate of inflammatory cells, fibrosis and microvessels [1] is a prototypical disease for studying the interplay between the tumour cell and its microenvironment.

- Secondly, the young age of patients and high curability provides the possibility of learning more about secondary complications of chemo- and radiotherapy and how to tailor treatment for the individual [3].

- Thirdly, an immune deficiency, possibly inherited or environmental, may help in understanding the interplay between the immune system, tumour growth, and tolerability to treatment [9, 10].
Fourthly, as a radio- and chemosensitive disease, and a disease with well defined target antigens in both tumour cells and surrounding cells [3], HL is well-suited for exploring alternative treatments, conventional, and targeted therapy, alone or in combinations. Many patients are young and can tolerate intense experimental approaches, if needed.

This thesis concentrates particularly on the first and second aspects. Papers I–III deal with negative prognostic factors, and Papers IV and V with the interaction and understanding of the role and presence of the surrounding eosinophils and mast cells.

History and aetiology

In 1832, Thomas Hodgkin described seven patients with enlarged lymph nodes [11], and in some of these patients the diagnosis of HL was later confirmed. In 1898, Carl Sternberg described the giant cell characteristic of HL tumour cells and at the same time Dorothy Reed observed and pictured cells with prominent nucleoli, which gave the name to the tumour cell population: Hodgkin and Reed-Sternberg cells [12, 13]. Dorothy Reed also described an association with tuberculosis and early after HL was described, a suspected infectious aetiology of the disease was postulated [3].

Despite this early suspicion, the aetiology of HL is still basically unknown, even though infection with Epstein-Barr virus (EBV) probably is important for a subset of patients, possibly through triggering chronic inflammation [14]. About 20–40% of HL cases harbour EBV-DNA in the HRS cells, and viral DNA is present in a monoclonal population of the HRS cells, implying that EBV was present before clonal expansion occurred in these cases [15-17]. Immuno-deficient individuals have an increased occurrence of HL, and these patients’ tumours more often harbour EBV [18]. A high prevalence of EBV-positive HL in childhood, might be attributed to a primary EBV infection and in older adults, caused by the loss of immunological control of a latent infection [19].

It is currently considered that EBV positive and EBV negative HL have different aetiologies [19, 20]. An infectious aetiology of EBV negative HL is suspected but no infectious agent has yet been identified. An underlying immune dysregulation is speculated as the cause of HL, for example a 3- to 5-fold increased risk of HL is observed in patients with rheumatoid arthritis (RA) and systemic lupus erytomatosis (SLE), even though some non-HL (NHL) cases might have been misclassified as HL possibly affecting these risk estimates [21]. First degree relatives have an increased risk of developing HL, probably due to an interplay of genetic susceptibility and shared environmental exposure [3].
Clinical presentation – from diagnosis to late complications

Diagnosis

Most patients with HL are diagnosed with enlarged lymph nodes. The disease can start in any lymphatic region, but the most common sites are within the cervical region and in the mediastinum [8]. In addition to medical history and physical investigation, the diagnostic procedures include a tumour biopsy, computed tomography from the neck to the pelvis, and laboratory investigations. A bone marrow biopsy is needed if the patient has advanced disease [22]. FDG-positron emission tomography (PET) can be used, although the exact role of PET is yet to be determined [23]. PET positivity or negativity after two courses of chemotherapy is today in many studies the bases for deciding the type and number of chemotherapy courses to continue with, making both an initial PET and one, after two courses, of value [24].

Staging and negative prognostic factors

The patient is staged according to the Ann Arbor classification [25], modified in Cotswolds [26] (Table 1). The staging provides the basis for initial treatment decisions. The different stages can be further subgrouped according to risk factors. Risk factors differ between the early and advanced stages and vary slightly between countries.

The risk factors are all known negative prognostic factors. Several other negative prognostic factors for HL have during the years been described, but many of these are not used when deciding treatment. However, the search for prognostic factors has different purposes. Firstly, to be able to predict outcome and to know which patients should be treated in a particular way, and secondly, to inform researches about the potential relevance of a factor for the aetiology, pathogenesis or clinical course of the disease. Although these two purposes overlap, they put different demands on methodology and the clinical material investigated.
Table 1. Classification according to Ann Arbor and additional subgrouping

<table>
<thead>
<tr>
<th>Classification</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Involvement of one lymph-node region</td>
</tr>
<tr>
<td>Stage II</td>
<td>Involvement of two or more lymph-node regions on one side of the diaphragm</td>
</tr>
<tr>
<td>Stage III</td>
<td>Involvement on both sides of the diaphragm</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Disseminated extra nodal involvement (always if liver or bone-marrow is involved)</td>
</tr>
</tbody>
</table>

A
No B symptoms

B
Fever >38°C, weight loss (>10% in 6 months) and/or night sweats

Bulky
A tumour ≥10 cm or a mediastinal tumour larger than 1/3 of the thorax diameter at the Th 5–6 level on a chest X-ray

E
Involvement of a single extra nodal site, or contiguous or proximal to known nodal site

CS
Clinical stage

PS
Pathological stage = staging laparotomy with splenectomy performed (not used today, but occasionally in some patients in I–III)

Supra
Disease above the diaphragm

Infra
Disease below the diaphragm

Early stages
I–IIA

Intermediate
IIB (discussed below), I–IIA with risk factors, IIIA

Advanced
III–IV

Histopathology

HL tumours are subclassified according to histopathology, either as nodular lymphocyte predominant HL (NLPHL) or classical HL (CHL). CHL is further divided into nodular sclerosis (NSHL), mixed cellularity (MCHL), lymphocyte-rich (LRCHL), and lymphocyte-depleted (LDHL).

NLPHL has a different immunophenotype than CHL, and the tumour cells are CD20-positive but lack CD30 antigens.

The different subtypes of CHL differ in many aspects but the few tumour cells, HRS-cells, have the same immunophenotype and often express CD30 and CD15. Hodgkin (H) cells are mononucleated and Reed-Sternberg (RS) cells are large, sometimes bi- or multinucleated cells, and they are scattered in a rich inflammatory infiltrate. The surrounding tissue is known for extensive interaction with the HRS cells. Briefly, NSHL accounts for approximately 70% of CHL and is characterised by the presence of collagen bands. These fibrous bands divide the tissue into nodules, giving it a very characteristic picture. In the cellular areas, the few malignant cells are surrounded by small lymphocytes and many other non-neoplastic inflammatory cells [1]. The inflammatory cells together with the HRS cells contribute to the fibrosis [27]. Most patients with NSHL present with stage II disease and are often younger than those with MCHL. MCHL is characterised by scattered HRS cells in a diffuse mixed inflammatory
background and is often associated with elderly patients and those with EBV positive tumour cells [3]. LRCHL has a background characterised by an abundance of small lymphocytes. The patients with LRCHL rarely present with mediastinal involvement or bulky disease. LDHL is rich in HRS cells and/or depleted in non-neoplastic lymphocytes: it is now a rare subtype and many cases of previously diagnosed LDHL are recognised as lymphomas other than HL [1].

The same histologic type is usually present in the initial and relapsed specimen, although progression from one type to another occasionally occurs [3]. There is a tendency for the number of eosinophil granulocytes, histiocytes, and Hodgkin cells to increase in relapsed tumours. The classification is important for the diagnosis, but does not influence treatment decision, except in the case of NLPHL subgrouping [28-30]. Apart from the tumour cells per se being important, and the age and EBV association, little is known about the determinants for histology.

Treatment

Treatment in an international perspective

Treatment recommendations for HL are constantly being revised. Therapy varies throughout the world, but in general patients in low stages (I and II) are treated with a combination of a few courses of chemotherapy (CT) and radiotherapy (RT) and patients with advanced stages (III and IV) are treated with several courses of CT. In recent years, the treatment has become more and more individualised, with the aim of providing enough to cure, but as little as possible to avoid complications [23].

Radiotherapy

RT was the first curative treatment, based upon principles developed by Peters in 1958 and Kaplan in 1966 [31, 32]. In their protocols RT was given to large fields (mantle fields, “inverted Y fields” or both), covering the tumours and unaffected tissues nearby. To reduce unwanted side-affects in later years, RT was reduced to involved field (IFRT), meaning only coverage of the involved lymph node region. Today, RT to only the involved lymph node (INRT) is tested [33].

Chemotherapy

Single cytostatic drugs have an effect on HL, but it was not until the mid 1960s when a combination of 4 drugs, mechlorethamine, vincristine, procarbazine and prednisone (the MOPP regime) revolutionised the treatment of advanced HL [34]. In 1975, an alternative regime of ABVD was developed (Table 2) that contains “non-cross resistant” drugs and shows a similar effect as MOPP [35]. In later studies, ABVD revealed less
secondary complications than MOPP or MOPP/ABVD [36]. Alternative regimes have later been developed, such as BEACOPP (Table 3) [37], and the CT mostly used today are ABVD and BEACOPP. In 2003, BEACOPP showed survial benefit for advanced stages compared to COPP/ABVD [38].

Table 2. **ABVD chemotherapy**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Group of drug</th>
<th>Major side effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin (A)</td>
<td>Anthracycline</td>
<td>Haemotologic, cardiac</td>
</tr>
<tr>
<td>Bleomycin (B)</td>
<td>Other</td>
<td>Pulmonary</td>
</tr>
<tr>
<td>Vinblastine (V)</td>
<td>Vinca-alkaloid</td>
<td>Haematologic</td>
</tr>
<tr>
<td>Dacarbazine (D)</td>
<td>Alkylating</td>
<td>Haematologic</td>
</tr>
</tbody>
</table>

Table 3. **BEACOPP chemotherapy**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Group of drug</th>
<th>Major side effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleomycin (B)</td>
<td>Other</td>
<td>Pulmonary</td>
</tr>
<tr>
<td>Etoposide (E)</td>
<td>Topoisomeras II inhibitor</td>
<td>Haematologic</td>
</tr>
<tr>
<td>Doxorubicin (A)</td>
<td>Anthracycline</td>
<td>Cardiac, haematologic</td>
</tr>
<tr>
<td>Cyclophosphamide (C)</td>
<td>Alkylating</td>
<td>Haematologic, nausea</td>
</tr>
<tr>
<td>Vincristine (O)</td>
<td>Vinca-alkaloid</td>
<td>Nausea, neuropathy</td>
</tr>
<tr>
<td>Procarbazine (P)</td>
<td>Alkylating</td>
<td>Haematologic</td>
</tr>
<tr>
<td>Prednisone (P)</td>
<td>Cortico-steroid</td>
<td>Endocrine-diabetes etc</td>
</tr>
</tbody>
</table>

**Other treatment**

Autologous stem-cell transplantation (ASCT) is used for primary refractory patients or patients experiencing an intermediate- and poor-risk first relapse. Single ASCT is appropriate for intermediate-risk patients and tandem ASCT for primary refractory and relapsed patients with many risk factors [39]. Allogenic stem-cell transplantations are also used. Treatment with antibodies (rituximab) is included in clinical studies in the primary treatment, as in the German HD18 study, in addition to CT and RT [40]. New targeted therapies are being tested for chemoresistant and multiple relapsing patients [3].

**Treatment currently used in Sweden (2009)**

**Early stages**

In stages I or II without B-symptoms or other risk factors (Table 4), two courses of ABVD (CHOP\(^1\)-21 for patients >70 years) followed by IFRT of 30 Gy is recommended. For patients with one or more risk factors, four courses of ABVD followed by IFRT of 30 Gy are recommended. Patients with NLPHL in stage IA, without risk factors, are only treated with RT.

\(^1\)CHOP=cyclophosphamide, adriamycin, vincristine, prednisone
Table 4. Risk factors for early stages

<table>
<thead>
<tr>
<th>CHL</th>
<th>NLP HL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulky</td>
<td>Bulky</td>
</tr>
<tr>
<td>&gt;2 lymph node regions involved</td>
<td>&gt;2 lymph node regions involved</td>
</tr>
<tr>
<td>(infra*)</td>
<td></td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate ≥50</td>
<td>Erythrocyte sedimentation rate ≥50</td>
</tr>
<tr>
<td>2 lymph node regions apart from each other</td>
<td></td>
</tr>
</tbody>
</table>

* If infradiaphragmal disease the risk factor is (stage II or stage I in abdomen or pelvis) instead

Advanced stages

For stages IIB–IV, treatment recommendations for patients <60 years with 0–2 risk factors according to the International Prognostic Score (IPS) (Table 5) are 6–8 courses of ABVD. For those with ≥3 risk factors in stages III and IV, a randomisation between eight ABVD or four dose-intensive BEACOPP and 4 standard BEACOPP is used (part of a multicentre study in the European Organisation for Research and Treatment of Cancer (EORTC)). For patients with stage IIB bulky disease or patients in stages III and IV with IPS>2, outside the study, 6–8 courses of BEACOPP14 (esc) is recommended. For patients between 60 and 70 years old, 6–8 ABVD is used and for patients older than 70 years, the chemotherapy is six CHOP14/21.

Table 5. Risk factors for advanced stages as described by Hasenclever & Diehl, 1998 [41]

<table>
<thead>
<tr>
<th>International Prognostic Score (IPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
</tr>
<tr>
<td>&gt;45 years of age</td>
</tr>
<tr>
<td>Stage IV disease</td>
</tr>
<tr>
<td>Haemoglobin &gt;105g/L</td>
</tr>
<tr>
<td>S-Albumin &lt;40g/L</td>
</tr>
<tr>
<td>S-leukocyte count (WBC) &gt;15x10⁹ g/L</td>
</tr>
<tr>
<td>B-lymphocyte &lt;8% or &lt;0.6x10⁹/L</td>
</tr>
</tbody>
</table>

Planned study for advanced stages

Cancer centres in Sweden plan to join the RATHL study (Response adapted therapy of Hodgkin Lymphoma). The study starts with 2ABVD, and then a PET scan should be performed. If the PET scan is negative, a randomisation between 4 ABVD and 4 AVD will be done and if the PET is positive, 4 BEACOPP 14 (3 BEACOPPesc) will be given, followed by a new PET scan. If this PET scan is negative, two additional BEACOPP14 (or 1 BEACOPP esc) will be given, and if still positive, RT or salvage therapy should be introduced.
Treatment protocols at other centres for patients in stage IIB
Stage IIB has throughout history been considered an early/intermediate stage. This group of patients can have very different clinical presentations, from two small locations and a single B-symptom to a large tumour burden together with severe B-symptoms, deranged laboratory parameters, and extranodal disease. As a result, staging, risk factor judgement, and treatment can vary. Before initiating the retrospective study I, a general impression among clinicians in Sweden was that prognosis appeared poor for patients in stage IIB, and there were uncertainties whether the patients should be included among early/intermediate or advanced stages, and it was unsure whether treatment recommendations were sufficient. Stage IIB is still under continuous investigation because of its divergent presentations and the difficulties in deciding an appropriate treatment remain. The EORTC and German Hodgkin Study Group (GHSG) have different study protocols for their stage IIB patients (Table 6).

Targeted therapy
A range of new drugs is under investigation for treatment of relapsed and refractory HL. Except for rituximab, none has yet been introduced into primary treatment. Rituximab, against CD20 is promising for NLPHL [42, 43] and is currently under investigation in CHL (HD18). Monoclonal antibodies are tested in patients with refractory disease; however, antibodies against CD30, the antigen present in all tumour cells, only have a complete remission rate around 10% [44]. Monoclonal antibodies against different antigens linked with radioimmunoconjugates or potent toxins have shown promising results [45, 46]. An immune modulator (IMiD) lenalidomide, known to enhance immune cell mediated killing, mTOR inhibitors (RAD001) and different H-DAC inhibitors have displayed early promising results.
<table>
<thead>
<tr>
<th>Study name</th>
<th>Age</th>
<th>Stage/risk factor</th>
<th>Treatment (standard arm=A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHSG, HD 13(^1)</td>
<td>18–75</td>
<td>IIB no RF*</td>
<td>2 ABVD + 30 Gy IFRT</td>
</tr>
<tr>
<td>(early stages)</td>
<td></td>
<td></td>
<td>2 ABV + 30 Gy IFRT (closed)</td>
</tr>
<tr>
<td>(to be replaced by HD16)</td>
<td></td>
<td></td>
<td>2 AVD + 30 Gy IFRT #</td>
</tr>
<tr>
<td>GHSG, HD 14(^1)</td>
<td>18–60</td>
<td>IIB ≥ 1 RF*</td>
<td>4 ABVD + 30 Gy IFRT</td>
</tr>
<tr>
<td>(intermediate)</td>
<td></td>
<td></td>
<td>2 BEACOPP esc + 2 ABVD + 30 Gy IFRT €</td>
</tr>
<tr>
<td>GHSG, HD 18(^1)</td>
<td>18–60</td>
<td>IIB Bulky mediastinum or E</td>
<td>A/C: 8 BEACOPPesc + 30 Gy RT if PET pos after last course</td>
</tr>
<tr>
<td>(advanced)</td>
<td></td>
<td></td>
<td>B/D: 4 BEACOPPesc if PET neg after course 2, 8 BEACOPPesc + Rituximab (course 4–8) if PET pos after course 2 + RT if still PET pos after last course</td>
</tr>
<tr>
<td>EORTC, H10(^2)</td>
<td>15–70</td>
<td>II (favourable=F) □ (unfavourable=U)</td>
<td>(F) 3 ABVD + 4–6 week INRT</td>
</tr>
<tr>
<td>(intermediate)</td>
<td></td>
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<td>(U) 4 ABVD + 4–6 week INRT</td>
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<td>(F/U) 2 ABVD, PET neg + 2/4 ABVD</td>
</tr>
<tr>
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<td></td>
<td>(F+U) 2 ABVD, PET pos + 2BEACOPPesc+4–6 week INRT</td>
</tr>
<tr>
<td>SWEDEN(^3)</td>
<td>18–70</td>
<td>IIB IPS $\geq$ 0–2</td>
<td>6–8ABVD</td>
</tr>
<tr>
<td>(advanced)</td>
<td>18–60</td>
<td>IIB IPS $\geq$ 2 or Bulky</td>
<td>6–8 BEACOPP14</td>
</tr>
</tbody>
</table>

* GHSG: Risk factors (RF): Bulky, E, ESR>50 or ESR>30 and B symptom, ≥3 nodal areas
\(\□\) EORTC: Favourable: ≤3 nodal areas, <50 yrs, ESR<50 or ESR<30 and B symp, Not bulky Unfavourable: ≥4 nodal areas, ≥50 yrs, ESR>50, ESR>30 and B symptom, Bulky
\(\$\) Sweden: Risk factors (RF) for advanced stages (IIB–IV) = International Prognostic Score
# According to an interim analysis, AVD is as good as ABVD
€ An interim analysis shows 2BEACOPPesc to be more effective than 2ABVD regarding OS
\(^1\)www.ghsg.org [40] \(^2\)www.eortc.be [47] \(^3\)www.roc.se [22]
Prognosis

Initially a deadly disease, HL is now a curable disease for most patients, with a 10-year overall survival (OS) of about 95% for early stages [23]. The prognosis for advanced stages is improving; with a 67% freedom from treatment failure (FFTF) and 79% OS for patients treated with standard regimes in international materials [48]. Advanced-stage patients treated with dose escalated BEACOPP have a FFTF of 85% after 7 years, and an OS of 90% after 7 years [48]. However, the long term effects of the new regimes for advanced stage HL are still unclear [48]. In Sweden, there has been a continuous improvement through the decades from a relative 10-year survival of about 30% for those (males and females 0-89 years of age) diagnosed in the 1960s and to 80-85% (6 year) during the mid 1990s [49]. The survival worldwide and in Sweden improved even further in the year 2000–2004 [50]. Females have in those materials a slightly better survival than males.

Late complications

Fifteen years after primary treatment for HL, the risk of death from other causes exceed the risk of death from HL. Secondary malignancies and cardiac disease are then the primary causes of death in patients cured of HL. The major categories of secondary cancers are leukaemia and solid tumours. The risk for developing leukaemia is largely dependent upon the amount of alkylating agents given [7] and is most prominent during the first 10 years after diagnosis [51]. The risk of developing a NHL is likely related to the underlying immunosuppression in HL patients [7, 52].

The risk of developing solid tumours increases every year for at least 25 years after the initial treatment [7]. Lung cancer represents the most frequent solid tumour, and both alkylating agents and RT increase the risk [4]. These two treatments have an additive risk and smoking multiplies this risk [4]. Breast cancer is the most frequent solid tumour among women, and the RT volume involving the breasts is associated with this increased risk. Treatment with alkylating agents reduces the risk of developing breast cancer, despite its carcinogenic properties, probably because it induces earlier menopause in treated women. Hormone stimulation appears important for the development of radiation-induced breast cancer [5]. In addition, the risk for other malignancies increases, particularly in patients who received their first treatment for HL at a younger age [7, 51].

Other secondary complications in HL-treated patients are acute myocardial infarction, congestive heart failure, thyroid failure, pulmonary dysfunction, and infertility [6, 53].

It is important to reduce treatment to the lowest amount possible, but also to inform cured HL patients who smoke about the importance of smoking
cessation [4]. The recommendations of how surviving HL patients should be cared for is a continuing discussion.

Connecting the clinical presentation with biology

Patients still die from HL, including young people. The ultimate goal must be to cure all patients without giving them severe, and sometimes fatal treatment side effects. In order to do this more needs to be known about the biology of the disease; so new therapeutic approaches can be found and the current approaches can be applied optimally. Identification of prognostic factors helps determine areas of importance for future studies and the optimal way to treat patient. Targeted therapy can move the focus of the conventional view of negative prognostic factors towards predictive factors of which targeted therapy to use. To determine the predictive factors, knowledge about biology is essential.
Inflammation and cancer

In the review from year 2000 “The Hallmarks of Cancer” Hanahan and Weinberg propose that tumours should be considered as a complex tissue, where the tumours cells have recruited and subverted normal cell types to serve as active collaborators. The tumour is now considered to consist of a complex network of tumour cells, inflammatory cells, fibroblasts and microvessels [54]. Each stage of cancer development is regulated by the immune system, where full activation of adaptive immune cells may result in eradication of malignant cells, but chronic activation actually enhances tumour development [55].

Innate immune cells, such as dendritic cells, macrophages, mast cells, neutrophils, basophils and eosinophils are the first line of defence against pathogens and other “danger” signals. B- and T-lymphocytes are members of the adaptive immune system and display highly specialised elimination of pathogens. Adaptive immune cells are activated by innate cells [56].

HL tumours are characterised by very few malignant cells, the HRS cells, surrounded by a massive infiltrate of inflammatory cells, such as lymphocytes, eosinophils, neutrophils, histiocytes, plasma cells, mast cells, macrophages, and fibroblasts [1].

Possible interactions between HRS cells, mast cells and eosinophils in HL are described in the following sections and summarised in (Figure 1.)

![Figure 1. Interactions between HRS cells, eosinophils and mast cells.](image)
Hodgkin and Reed-Sternberg cells

The mono-nucleated Hodgkin cell and the multi-nucleated Reed-Sternberg cell (HRS) represent 0.1–10% of the cell population in the tumour tissue [1]. HRS cells are derived from B-cells at various stages of development and in rare cases from T-cells. This was proven when clonal rearranged immunoglobulin genes were amplified from single HRS cells that had been micromanipulated from HL biopsies [57]. Germinal centre (GC) or post GC B-cells are probably the precursors of HRS cells. HRS cells express proteins that are typical for various cell types, but B-cell lineage markers, such as CD20, B-cell receptor (BCR), or CD79a are rare on the surface of the cells, probably due to loss of B-cell specific transcription factors and/or epigenetic alterations of B-cell specific genes [3]. However, a small proportion of HRS appears to retain expression of B-cell specific genes. In the GC, there is a strong selection for cells presenting the BCR. Normal B-cells that loose their BCR go through apoptosis, but HRS cells evade apoptotic cell death and this eventually leads to systemic lymphoma disease [58]. Another feature of HRS cells is that they are genetically and chromosomally unstable [59]. Genetic lesions found in HRS cells involve members of two signalling pathways: Janus (jak)-stat and nuclear factor κB (NF-κB) [60]. HRS cells express CD30 in nearly all cases, and can be activated by stimulation of CD30 ligand (CD30L) [61-63]. However, conflicting data have been published regarding the role of CD30 in the activation of NF-κB and the need for ligand-binding for activity of CD30 [60].

Cytokines are potent proteins that serve as messengers for the immune system and are active in regulating immune and inflammatory responses, contributing to haematopoiesis, stimulating wound healing, and participating in other biological processes. Cytokines can act both in an autocrine manner on the same cell that produced the cytokine and in a paracrine fashion on surrounding cells (Figure 2). HL has an abnormal production of cytokines and cytokine receptors, where a constitutive activity of NF-κB and an altered Jak-Stat signalling pathway are part of the biological background of this production [3]. The presence of reactive cells in HL tissue is partly due to the production of specific cytokines or chemokines by HRS cells [64, 65].

![Figure 2. Autocrine and paracrine signalling.](image)
Postulated stem cells

The existence of cancer stem cells is currently under intense investigation in many malignancies [66]. The precursor of HRS cells is GC or post GC B-cells, and this excludes HRS cells being derived from haematopoietic stem cells [60]. However, this does not exclude the possibility that the transformation process to a tumour cell is acquired in a lymphoma precursor at an earlier differentiation stage, as proposed by Kuppers in 2009. He also discusses that a subset of HRS cells possibly has a higher proliferative and renewal potential than the majority of the tumour cell clone, and as such can be defined as cancer stem cells [60]. Both in an old study [67] and in a recent one [68], CD19 and CD20 expression of a small subpopulation of tumour cells in the cell line L428 was found. CD19 positive cells were also found in blood from HL patients in the recent study. These cells are speculated as tumour stem cells, as they grow easily in cell culture. However, the definite role of tumour stem cells in HL is not elucidated and animal models are probably needed for determining this.

The combination of the intrinsic characteristics of stem cells and their microenvironment determines the properties and defines the potential of normal stem cells [69]. Postulated cancer stem cells in other malignancies are associated with a micro-environmental niche, indicating that there may be important interactions between the stroma and postulated cancer stem cells [70].

Mast cells

Mast cells originate from haematopoietic progenitor cells within the bone marrow that circulate in the blood and are recruited into the tissue, where they acquire their mature phenotype. Mast cells exist in almost all vascularised tissue but are especially numerous in the skin, respiratory tract and gastrointestinal tract. On the cell surface, mast cells have a high affinity receptor for IgE and are primarily associated with diseases such as atopic asthma, allergic rhinitis and atopic dermatitis. They also play a role in many other diseases [71, 72]. Mast cells are largely dependent on stem cell factor (SCF) as the promotor of their development [73]. In addition to SCF, other factors such as IL-9 are suggested to contribute to their development: IL-9 increases the quantity and size of mast cell colonies grown with SCF [74]. Upon activation, mast cells secrete preformed mediators, for example histamine and tryptase that they have stored in granules. They also synthesise de novo and secrete several cytokines, chemokines, and lipid mediators [75].
Mast cells in tumours

The presence of mast cells in tumour tissue is not a new finding [76], but their role in tumours is still largely unknown. Mast cells might be recruited by the tumour for its own benefit, accumulate as a reaction to the tumour, or be innocent bystander cells. Mast cells have stimulatory effects on cancer growth, such as vasodilation, stimulation of neo-angiogenesis, tissue damage and metastatic stimulation, and has inhibitory effects on tumour growth, for example stimulation of an anti-tumour adaptive response and vasoconstriction. However, mast cells accumulate around tumours and it appears they can either promote or inhibit tumour growth depending on local stromal conditions existing in the microenvironment of different tumours [71, 77].

![Figure 3. Hodgkin lymphoma tissue with scattered mast cells (in brown).](image)

Mast cells in HL

In HL-affected tissue, mast cells are present in all subtypes (Figure 3), but predominantly in NSHL [78]. The chemokine CCL5/RANTES produced by HRS cells has a possible role in the accumulation of mast cells in HL [79]. A high mast cell infiltration in HL-affected tumours correlates with worse DFS, high white blood cell count, and low B-Hb in one study [78]. Mast cells express functional CD30L and are the predominant CD30L-positive cells in HL [80]. The activation of HRS cells by CD30L may result in the proliferation of tumour cells [61-63]. After stimulation with CD30, mast cells are also stimulated to de novo synthesis of chemokines, which is most
prominent for IL-8, a known angiogenesis-stimulating cytokine [77]. The relation between presence of mast cells and degree of angiogenesis in HL is therefore of great interest to investigate.

Eosinophils
The eosinophil is a bone-marrow derived leukocyte that is present most frequently in the gastrointestinal tract and lymphatic tissue of healthy individuals and is measurable in peripheral blood. Eosinophil development from myeloid progenitors is primarily regulated by cytokines such as IL-3, IL-5, and granulocyte macrophage-colony stimulating factor [81]. Eosinophils are only transiently present in the peripheral blood and is primarily a tissue-dwelling cell. The recruitment of eosinophils into inflammatory sites involves a number of cytokines [82]. Eosinophils probably evolved as protection against parasites and are thought to primarily be associated with asthma and parasitic infections [81]. Eosinophils contain granules with mediators belonging to three different families: 1. major basic protein (MBP) 1 and 2 from the C-type lectin family; 2. eosinophil peroxidase (EPO) from the peroxidase family; and 3. eosinophil-derived neurotoxin (EDN, RNAase2) (also called eosinophil-protein X (EPX)) and eosinophil cationic protein (ECP, RNAase3) which belongs to the ribonuclease family. These mediators are among the most basic substances in mammals and are toxic to parasites, and they damage human body tissue during disease.

Eosinophils in tumours
Eosinophils are associated with the inflammatory response within tumours where they may have two fundamentally different roles. Firstly, they may potentially limit tumour growth by performing destructive functions as well as recruiting and activating other leukocytes. Secondly, eosinophils may be immunoregulators and remodel cells that suppress immune responses and promote tumour cell proliferation. The definite role of eosinophils in tumours is unknown and appears to be different in different tumours [83, 84].

Eosinophils in HL
In HL, some cases show prominent eosinophilia within tumours (Figure 4) [85]. Tumors heavily infiltrated with eosinophils have a poor prognosis [85, 86]. However, this correlation has been questioned [87-89]. CD30 ligand is expressed on eosinophils [61], even though the predominant CD30L-expressing cell in HL is considered to be the mast cell [80]. The CD30-CD30L interaction can stimulate HRS cell proliferation, which maybe explains the poor prognosis in eosinophil- and mast cell-rich cases of HL [61-63]. As eosinophils are present in HL tumours, in some cases in large
amounts, the reasons behind this and their influence on tumour behaviour need to be determined.

**Figure 4.** A Reed-Sternberg cell surrounded by eosinophils (in pink).

### ECP from eosinophils

ECP, one of the eosinophils granulaproteins, is a protein with many functions but is mostly known for its cytotoxic effect [90]. The cytotoxic ability of ECP is thought partly to be because it creates voltage insensitive pores in the agent with which it has contact. ECP is cytotoxic to parasites, bacteria, viruses, cancer cells and sometimes to normal tissue cells. ECP has RNase activity [81], but this activity is considerably lower than from one of the other granula proteins, EDN/EPX. Several Single Nucleotide Polymorphisms (SNPs) exist in the ECP gene, in this thesis the SNP ECP434(G>C) was studied the most. The different genotypes of ECP434(G>C) polymorphism are ECP434GG, ECP434GC, and ECP434CC. In a Scandinavian population, the genotypes ECP434GG and GC are the most common: ECP434CC is rare [91]. This polymorphism results in an amino acid substitution from arginine to threonine at position 97 of the altered protein.

- **ECP434GG** gives the protein ECP97arg
- **ECP434GC** gives a mixture of ECP97arg and ECP97thr
- **ECP434CC** gives the protein ECP97thr
ECP97arg from individuals with ECP434GG is more cytotoxic in model systems than ECP97thr [92]. A lower level of glycosylation of the ECP97arg protein further potentiates the cytotoxic properties of ECP when explored in a cell line [92]. Because of its different functions on the protein level this polymorphism is of interest in the study in diseases. ECP562(G>C) is not a coding polymorphism; however, the polymorphism is probably in a regulatory element of the ECP-gene that governs how much ECP is produced by the eosinophils [84].

ECP is present in the sera of patients diagnosed with HL and correlates to the number of eosinophils in the HL tumours. High serum-ECP levels also correlate to high ESR, NSHL histology, advanced stage, and bulky disease [93]. ECP is involved in maintaining fibrosis in other diseases [94, 95], and more needs to be understood about its role in fibrosis-rich NSHL cases. The effect of ECP on HL cell lines has previously not been reported.

The cytokine IL-9 and its receptor IL-9R
Interleukin-9 (IL-9) is a multifunctional cytokine and has, in addition to its activities in immune and inflammatory responses such as asthma [96], a suggested role in promoting oncogenesis [97]. Among malignant tumours, IL-9 is almost exclusively related to HL (Figure 5) and anaplastic large cell lymphomas [98]. The main source of IL-9 is activated Th2-lymphocytes, but other cell types including mast cells can produce IL-9 [97, 99]. The HL cell lines HDLM-2 and KM-H2 express IL-9 and respond to IL-9 stimulation. Based on this, an autocrine loop is postulated [100]. IL-9 also synergises with stem cell factor (SCF) to promote the growth of cultured HRS cells of the KM-H2 cell line [101]. An increased serum level of IL-9 is found in about 40% of patients with HL [102, 103] and a correlation between serum levels and expression in tumours indicates that HRS cells are the source of IL-9 in serum [102].

The IL-9 receptor is part of the haemopoietin receptor superfamily and is expressed as both a soluble and as a membrane-bound form (Figure 5) [104]. Many effects from IL-9 on murine eosinophils and murine and human mast cells are described [74, 104, 105]. Consequently, the effects of IL-9 on the HL inflammatory infiltrate (mast cells and eosinophils) and its importance for prognosis are of interest.
Angiogenesis

Angiogenesis is the formation and subsequent stabilisation of new vessels from pre-existing blood vessels [106, 107]. Angiogenesis has a well established role in the development of solid tumours [107, 108], and a potential role in lymphomas has been postulated [108]. The mechanisms behind angiogenesis are a complex interplay between the malignant cells and their surrounding tissue and are as yet incompletely understood.

Angiogenesis and mast cells

Mast cells are associated with angiogenesis both in solid tumours and in haematological malignancies, such as B-cell NHL and multiple myeloma [109]. Mast cells express several factors that can affect angiogenesis both directly and indirectly. These factors are vascular endothelial growth factor (VEGF), basic fibroblast growth factor, transforming growth factor-beta (TGF-β), tumour necrosis factor-alpha, IL-8, histamine, tryptase, matrix metalloproteinase-9, and heparin, as reviewed by Norrby [110].

Angiogenesis in HL

There is limited information on the role of angiogenesis in HL. VEGF, a factor that regulates multiple endothelial cell functions is expressed by HRS cells in 70% of HL lymph nodes and HL cell lines [111]. High levels of VEGF in sera, both pre- and post-therapy, are independently predictive of survival in patients with HL in one study [112], but not in another more recent study [103]. Other angiogenic factors, such as hepatocyte growth factor and basic fibroblast growth factor, decrease in HL patients after treatment [113]. A high microvessel density (MVD) is a negative prognostic
factor in HL [114]. Thus a part of this thesis was to determine if this was true in another material and in particular if mast cell number was related to angiogenesis in HL (Figure 6).

![Hodgkin lymphoma tissue with multiple microvessels.](image)

**Figure 6.** Hodgkin lymphoma tissue with multiple microvessels.

**Angiogenesis inhibitors in the treatment of HL**

In routine clinical practice, angiogenesis inhibitors are not yet utilised in the primary or secondary treatment of HL. Although they likely have been tried in patients with refractory HL, no data from prospective trials is available. Clinical trials are conducted on drugs with antiangiogenic activities as part of their mechanisms, such as lenalidomide (Revlemid®). In addition to the antiangiogenic activity lenalidomide exhibits a range of clinical properties; being an immunmodulatory drug; such as activation of natural killer (NK)-cells and T-cells, being anti-proliferative for tumour cells and pro-erythropoietic [115, 116]. Low dose oral metronomic CT is considered to have antiangiogenic properties in NHL, as it prevents mobilisation of endothelial progenitor cells into the blood. Low dose oral metronomic CT could be relevant for HL as well [117, 118].
Other cell types

**T-lymphocytes**
The majority of non-malignant lymphocytes in CHL are comprised of T-cells and the cells in closest vicinity to the HRS cells are almost always positive for CD4 (helper T-cells): few CD8 positive (cytotoxic T-cells) or NK- cells are present in this area [3]. However, activated CD8 positive cells are described in the tissue, but are not immediately surrounding the HRS cells. Surprisingly, the presence of activated CD8 positive cells is associated with poor prognosis [119]. The T-cells in HL are in an anergic state; thus, they do not show their normal functions and are incapable of causing an effective immune response towards the tumour cells. This anergy is probably induced by the HRS-cells [3].

A third type of T-cells, the CD4CD25 positive (regulatory T-cells) are also present in the tissue, and a low number of these cells and a high number of cytotoxic T-cells relates to poor prognosis [120].

**Antigen presenting cells**
Gene clusters, identified by microarray technology, including overexpression of genes expressed by specific subpopulations of macrophages, plasmacytoid dendritic cells and of T-cells are related to an unfavourable prognosis. In tissue microarray (TMA), these results are confirmed for example for macrophages; conversely, genes involved in fibroblast function and chemotaxis, molecules expressed by antigen presenting cells and a specific subpopulation of B-cells are related to a favourable outcome [121].

**Fibroblasts**
Fibroblast and tumour cells show a large degree of heterogeneity. Fibroblasts are in other studies considered to have an important role in the supportive tissue of tumours [122]. The presence of tumour fibroblasts leads to faster tumour growth in breast cancer, by its link to recruitment of angiogenesis-stimulating cells [123]. The tumour stroma is involved in the process of metastasis and experiments on co-culture of breast cancer tumour cells and mesenchymal cells (used as model cells for tumour fibroblasts) caused an accentuated production of CCL5/RANTES that stimulated the tumour cells invasive ability [124]. In HL, fibroblasts are present, especially in NSHL histology. The development of fibrosis in HL is a process where both eosinophils and mast cells have a possible supportive role [27].

The microenvironment – an intricate balance

The microenvironment is important for disease development, disease progression, and prognosis, and consequently, for deciding treatment.
However it is an intricate balance, where particular cells can have both growth-supportive and growth suppressive roles.

Studies of prognostic markers from the microenvironment [125-127] might lose significance depending on treatment given for the disease. For example, in follicular lymphoma, macrophages, regulatory T-cells, and mast cells are prognostic markers when standard treatment of CHOP is administrated, but the prognostic importance disappears when rituximab is introduced. The same may apply to different treatment modalities for HL, for example, the negative prognosis of angiogenesis could disappear when angiogenesis inhibitors are introduced. The picture of the microenvironment could indicate target therapy depending on what cell types, cytokines, and vessels are expressed in the individual tumour. The exact activity of the different cell types is difficult to fully elucidate, and animal models are needed.

Aside from the tumour cells per se being important for the composition of the microenvironment, little is known about the determinants of its composition. The roles of genetic constitution, lifestyle, and other diseases have not been studied. Elucidation of the determinants of the microenvironment is important for both individual risk assessment and prevention strategies.
Overall aim

To study the clinical presentation, tumour cells and surrounding inflammatory cells in patients with HL in order to determine prognostic factors that could be used to specifically adjust treatment to the individual. Based on this knowledge, it may be possible to find aetiological explanations for the development of HL, and design new treatment strategies.

Specific aims

I To evaluate the treatment results of patients who presented with stage IIB, and to identify subgroups of patients in this stage, depending on their negative prognostic factors.

II To explore the relationships between the cytokine IL-9 and the number of mast cells and eosinophils in HL tumours, and to investigate how expression of IL-9 and the IL-9 receptor relates to clinical characteristics and prognosis in HL.

III To investigate the relation between number of mast cells and the microvessel count and further elucidate the prognostic implication of microvessel count in HL.

IV To investigate the effect of the eosinophil protein, ECP, on HRS cells in vitro.

V To investigate the associations between selected environmental and constitutional factors, the histology, and the presence of eosinophils and mast cells in HL microenvironment.
Material and Methods

Patient materials

Patient samples and clinical data were retrieved from the database of the National Health Care Programme for HL, covering all patients diagnosed with HL, in 5 out of 6 health care regions in Sweden [128-130]. All patients in the database diagnosed between 1985 and 1994, below the age of 60 years and in stage IIB, were included in Paper I (n=99). In Paper II, diagnostic paraffin-embedded tissue blocks and clinical information were available for 131 patients in one of the regions, the Uppsala/Örebro health care region, between 1989 and 1994. In Paper III, 120 of those patients treated with curative intention were included (the reduced number was due to lack of tumour material in a few cases). For Paper V, a population-based study of 585 patients diagnosed in Sweden and Denmark between 1999 and 2002 were utilized [20, 131] and available tumour biopsies from 448 patients were investigated. In the studies of tumour material (Papers II, III and V), all diagnoses were re-evaluated using the WHO classification [1]. All studies dealing with patient material were ethically approved.

Treatments

Treatment in Paper I for stage IIB patients (1985–1994)

In Paper I, the treatment recommendation for patients (16–60 years) were 6-8 courses of CT, followed by IFRT in the case of initially bulky disease, slow tumour progression (30Gy), or residual disease (40Gy). The recommended CT was MOPP/ABVD\(^1\) and MOPP/ABV hybrid starting January 1994. If the patient was pathologically staged a reduction to two cycles of CT and mantle field RT could be done.

\(^1\) MOPP/ABVD = mechlorethamine, vincristine, prednisone, procarbazine/adriamycin, bleomycin, vinblastine, and dacarbazine


In Papers II and III, the treatment for patients with stages IA and PSIIA was EFRT (mantle field) only. If they had bulky disease, two courses of CT were given before the RT, it was the same for patients in PS I and PSIIB. For CS IIA, IB and IIB patients, 6–8 courses of CT ± RT were given. Patients with
PS IIIA had subtotal nodal irradiation either alone or preceded by two courses of CT, if they had bulky disease. The advanced stages (CS + PS IIIA + B, IV A and B) were treated with 6–8 courses of CT ± RT. The CT and RT were as described for stage IIB.


As the principles of primary treatment of HL have not changed radically during the past decade, the patients in Paper V were treated as described in the background section. Some patients were treated with MOPP/ABV combinations rather than only ABVD. FDG-PET was, however, not used as a prognostic marker to decide treatment for the patients in Paper V.

**Cell lines**

For Paper IV the HL cell lines utilised were HDLM-2 (*Figure 7*), a cell line of T-cell origin developed from pleural effusion of a male with NSHL (DSMZ, no ACC17) [132, 133]. The other cell lines described are of B-cell origin [134]. L428 came from a female suffering from NSHL [135] and KM-H2 from a patient originally with MCHL and later with LDHL [136]. All cell lines were cultured in RPMI supplemented with 10% heat inactivated foetal calf serum, glutamine, penicillin, and streptomycin. The small-cell lung cancer cell-line NCI-H69 [137] was used as a reference cell line and cultured under the same conditions.
Immunohistochemistry and evaluation

The lymph node tumour tissue used for patients in Papers II, III, and V was formalin-fixed, paraffin-embedded and sectioned in 3–4 μm thick sections.

In Paper II, cases were manually stained with polyclonal IL-9 and monoclonal IL-9R. The material was considered positive if more than 5% of the tumour cells were unequivocally stained in the cytoplasm (Figure 5).

In Paper III, microvessels were stained with CD31 in the Ventana ES immunostainer (Ventana Medical Systems, Inc., Tucson, USA). The estimation of the number of microvessels was with the Chalkely technique [138]. Three to five fields with the highest concentration of vessels (a hot spot) were counted and the average of the highest three counts in every case was used (Figure 6).

Immunostaining and counting of the mast cells (Figure 3) (Paper II, III and V) were with monoclonal G3-antibody recognising tryptase. The mast cells were counted in 10 randomly selected high power fields (HPF 400x, Paper II and III) and (200x magnification Paper V). The ocular had a lattice square net and the numbers of tryptase-positive cells within the net area were counted [78]. The stainings for eosinophil counting (Figure 4) were with haematoxylin-eosin, and 10 randomly selected HPFs were examined [85]. In NSHL cases, only cellular areas were counted.
Cytotoxic assay

After culture with native ECP or EPX a fluorometric microculture cytotoxicity-assay (FMCA) measured the survival index (SI) of cells from HL cell lines (Figure 8), Paper IV. ECP and EPX were purified from healthy blood donors, as described in [92]. ECP with different levels of glycosylation and purified gene products of the ECP 434(G>C) polymorphism were tested. Cells were cultured and seeded in 96-microtiter plates. ECP and EPX, diluted in NaAc buffer, were added to the wells. Wells containing only buffer served as controls. Plates were incubated for 72 hours before the addition of fluorescein diacetate (FDA). Fluorescence was read with a fluorescan, as described in [139]. The generated fluorescence is proportional to the number of cells with an intact plasma membrane and data is presented as SI %.

\[
SI = \frac{\text{mean fluorescence in test wells} - \text{fluorescence in 12 blank wells}}{\text{mean fluorescence in control wells} - \text{fluorescence in 12 blank wells}} \times 100(\%)
\]

![Figure 8. Description of FMCA assay.](image)

Flow cytometry

Cells from the HDLM-2 cell line were seeded in serum-free conditions in 24-well plates with 60 000 cells per well. Cells were cultured with 0.07μM of ECP (ECP-treated cells) or NaAc buffer (buffer-treated cells). After 72 h
incubation the cells were carefully retrieved from the wells and counted with a light microscope.

Five to ten thousand (5000–10 000) cells were incubated with FDA, PI or annexin V. Cells were stained with anti-CD30 and the corresponding irrelevant antibody.

In all analysis ECP and buffer treated cells were compared to each other. CD30 was used as a positive marker for HDLM-2 cells. Cell viability was measured as percentage of cells stained with FDA (described above), related to non-stained cells. Cell death was also analysed by DNA staining (PI) and staining of phosphatidylserine (an early event during apoptosis: annexin V).

ECP and EPX genotyping
DNA was extracted from punches of dried filter paper cards from blood from patients in Paper V. DNA were subjected to whole genome amplification with AmpliQ Genomic Amplifier Kit (Ampliqon, Denmark). A modified protocol described previously [140] was used. DNA samples were genotyped for ECP 434(G>C), ECP 562(G>C) and EPX 405(G>C) polymorphisms with 5’ nuclease allelic discrimination assay. The PCR is described in detail in Paper V. The genotypes were determined by allelic discrimination according to the ABI Prism 7000 SDS software.

Statistical methods
Survival curves were constructed according to Kaplan-Meier and statistics calculated with log-rank test. Disease-free survival (DFS) (used in Paper I) /Progression-free survival (PFS) (used in Paper II and III) were calculated from the day of diagnosis to relapse or death from HL. Patients who did not reach complete remission had a DFS/PFS of zero days. Hodgkin-specific survival (HLS) was calculated from the day of diagnosis to death due directly to HL or due to other reasons, yet persistent with HL. Regression models were used to investigate associations between histology, number of eosinophils and mast cells and potential predictors for paper V.
Results and discussion

I Bulky disease is the most important prognostic factor in Hodgkin lymphoma stage IIB.

In Sweden, since 1985 all adult patients (>16 years) with HL have been treated according to a National Health Care Programme. The results are continuously evaluated and modified. This study identified all patients in five out of six health care regions (16–60 years of age) with stage IIB between 1985 and 1994 in Sweden. In the material of 99 patients, 86 patients had supradiaphragmal disease, 13 had infradiaphragmal disease, 47 were men, and 52 were women. From this study, it was concluded that patients in stage IIB should generally be considered as having an advanced stage, except for a small subgroup of patients that could be considered as having an intermediate stage. The OS in all 99 patients was 65% after 10 yrs, and after 10 yrs the HLS was 73% and DFS 65%. HLS was worse for patients in stage IIB than for patients in stages I to IVA; however, bulky disease was overrepresented in stage IIB compared to the other stages [129, 130] (Table 7) and bulky disease was the only significant negative prognostic factor in this study (Figure 9).

Table 7. HLS in stage IIB in comparison to other stages in Sweden 1985–1992

<table>
<thead>
<tr>
<th>Study</th>
<th>Stage</th>
<th>Bulky disease</th>
<th>HLS after 10yrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molin et al 2003</td>
<td>IA–IIA</td>
<td></td>
<td>&gt;90%</td>
</tr>
<tr>
<td></td>
<td>PSIIIB</td>
<td></td>
<td>91%</td>
</tr>
<tr>
<td>Glimelius et al 2003 (I)</td>
<td>CSIIIB</td>
<td>56%</td>
<td>73%</td>
</tr>
<tr>
<td>Amini et al 2000</td>
<td>PSIIB /CSIIIA</td>
<td>28%</td>
<td>92%/86%</td>
</tr>
<tr>
<td></td>
<td>PSIIBB /CSIIIB</td>
<td>34%</td>
<td>93%/89%</td>
</tr>
<tr>
<td></td>
<td>IVA</td>
<td>43%</td>
<td>76%</td>
</tr>
<tr>
<td></td>
<td>IVB</td>
<td>36%</td>
<td>61%</td>
</tr>
</tbody>
</table>
For patients in stage IIB a worse DFS and OS were observed, compared to patients in ongoing studies in Europe and USA at the same time (Table 8).

Table 8. **Swedish results compared to other centres at the same time as Paper I**

<table>
<thead>
<tr>
<th>Group/Study</th>
<th>Stage and risk factors</th>
<th>Treatment</th>
<th>10yr FFTF</th>
<th>10yr OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHSG HD51</td>
<td>I and II ≥ 1 RF*, all IIIA</td>
<td>A: 2 COPP + ABVD + 30Gy EFRT + 10Gy B: 2 COPP + 2ABV +2IMEP + 30Gy EFRT + 10Gy</td>
<td>79% (both arms, 7yr)</td>
<td>88% (both arms, 7yr)</td>
</tr>
<tr>
<td>N=996</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EORTC H72</td>
<td>I, II supra Favourable**</td>
<td>A: 6 EBVP + EFRT B: 6 EBVP + IFRT</td>
<td>A 78% B 88%</td>
<td>A 92% B 92%</td>
</tr>
<tr>
<td>N=722</td>
<td>II supra Unfavourable***</td>
<td>A: 6 EBVP + IFRT B: 6 MOPP/ABV + IFRT</td>
<td>A 68% B 88%</td>
<td>A: 79% B: 87%</td>
</tr>
<tr>
<td>Stanford3</td>
<td>I, II Bulky + IIIA, IIIB, IV</td>
<td>Stanford V + 36Gy modified mantle</td>
<td>89% (5yr)</td>
<td>96% (5yr)</td>
</tr>
<tr>
<td>88–99, N=142</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweden4</td>
<td>IIB</td>
<td>6–8 MOPP/ABVD + IFRT, 2 MOPP/ABV + Mantle if PS</td>
<td>65%</td>
<td>65%</td>
</tr>
<tr>
<td>85–94, N=99</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* RF: Bulky (>5 cm), involved spleen, ESR>50 or ESR>30 + B symp, >2 nodal areas  
** Favourable: Stage II, not Bulky, no B symp and ESR <50 or B symptoms and ESR <30  
*** Unfavourable: Stage II, Bulky, B symp and ESR >30 or no B symp but ESR >50  
∞ Minor differences on how this endpoint is defined exist between the studies

FFTF=freedom from treatment failure; OS=overall survival; EFRT=extended field; IFRT=involved field; Mantel=Mantle field; IMEP=ifosfamide/methotrexate/etoposide/prednisone; EBVP=epirubicin/bleomycin/vinblastine/prednisone; Stanford V=vinblastine, doxorubicin/vincristine/bleomycin/mustard/etoposide/prednisone; supra=supradiaphragmal disease, ESR=Erythrocyte Sedimentation Rate

1 Sieber 2005 [141], 2 Noordijk EM 2006 [142], 3 Horning, SJ 2002 [143], 4 Paper I
The two European studies HD5 and H7 include patients in both stages I and IIA and have not reported in detail the results for patients in stage IIB. The Stanford protocol has high survival, despite the material consisting of more advanced stages than IIB; however, the follow-up is only 5 years. In the Stanford material, there had been no relapses or deaths among the 28 patients with stage II bulky mediastinum after three years; however, only 5 of the 28 patients had B-symptoms [144].

The treatment within the study groups differs between countries, although all countries have combined modality treatments with 4–8 courses of CT and either IFRT or EFRT. In the Stanford protocol, a more permissive view on RT is used than in Sweden, as patients with disease >5 cm in diameter are irradiated. However, the treatment principles are probably not the only explanation for poor survival in patients with stage IIB in Sweden.

The frequency of bulky mediastinal involvement was a major difference seen between the different studies and was overrepresented in the Swedish study (Table 9). Bulky mediastinal involvement in combination with B symptoms appears a reasonable explanation for the poor prognosis in the Swedish material.

Table 9. Percentage of patients with bulky disease in other studies compared to Paper I.

<table>
<thead>
<tr>
<th>Studies</th>
<th>Bulky≥10cm/Large mediastinal mass (percentage of total material)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHSG HD5</td>
<td>Arm A: 23%&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Arm B: 26%&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>EORTC H7</td>
<td>23% (whole material)</td>
</tr>
<tr>
<td></td>
<td>42% (unfavourable group)</td>
</tr>
<tr>
<td>Stanford V</td>
<td>46% (few patients with B-symptoms)</td>
</tr>
<tr>
<td>Sweden stage IIB</td>
<td>56% (whole material)</td>
</tr>
<tr>
<td></td>
<td>63% (supradiaphragmal disease)&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>51% had bulky disease, however this was defined as only a ≥5 cm large tumour. In the HD5 material, 23% in arm A and 26% in arm B had a large mediastinal mass

<sup>2</sup>All but one patient had bulky disease in the mediastinum

The other studies are prospective and randomised whereas the study in Sweden was retrospective and patients were treated according to a defined care programme. In prospective protocols at dedicated cancer centers’ participating in a trial, treatments are likely to be more clearly defined and followed more carefully. The lack of close adherence to details in the recommendations given routinely at many sites (and not possible to explore in a retrospective evaluation) could contribute to a less favourable outcome.

In the Swedish guidelines today, patients in stage IIB are all included among the advanced stages [22]. The GHSG includes stage IIB patients in protocols for early, intermediate, and advanced stages, depending on how many risk factors are presented (Table 6 in Background). However, all patients with bulky mediastinal disease are included among the advanced
stages. The EORTC studies still include stage IIB among the intermediate stages in the unfavourable group and they randomise between four ABVD + INRT in the standard arm, and add either four extra ABVD to the initial two ABVD, if they are PET-negative, or, two BEACOPPesc and INRT, if they are PET-positive (Table 6 in Background).

A small subgroup of patients in stage IIB could be identified as candidates for less intensive treatment (less than 6–8 ABVD), as in the ongoing EORTC H10 study (if they have an ESR below 30, less than four affected locals and non-bulky disease). Among the nine patients in Paper I who fulfilled all three criteria, there have been no relapses. The remainder of the patients should be considered as having an advanced stage and should also be treated according to having an advanced condition. As a consequence of this study, the current recommendation in Sweden is six courses of BEACOPP-14 for patients in stage IIBX [22]. In comparison with these current recommendations, the planned RATHL study (described in the background), will start with two ABVD and then continue with FDG-PET monitored treatment. This initial treatment start may be too weak for some individuals.

Treatment recommendations, whether in a national, regional, or local programme must continuously be quality-controlled. Resources must be available within the health care system for the quality assurance and quality control of the recommendations.

Bulky disease is usually characterised by a massive infiltration of fibrosis in a large tumour bulk, leading us to study the cytokines found in HL tumours as they are considered to contribute to fibrosis. The abnormal and unbalanced cytokine production in HL may be responsible for the B-symptoms observed in HL, although the exact cause of these symptoms is unclear [65, 145].
II IL-9 expression contributes to the cellular composition in HL

Since an autocrine stimulatory loop has been demonstrated for IL-9 and IL-9R for HRS cells [100], the relation of IL-9 expression to histopathological and clinical characteristics in 131 HL patients was investigated. Patients with IL-9 positive tumour cells were younger and more often females than patients with IL-9 negative tumour cells were. IL-9 expression was present in the cytoplasm of HRS cells in about 50% of cases, and expression of IL-9R was present in about 20% (Figure 5). IL-9 expression correlated to cases with a high number of eosinophils and mast cells in the tumours and to cases with the histology of NSHL. Therefore it could by hypothesised that IL-9, in addition to other cytokines produced by the HRS cells, have a stimulatory effect on eosinophils and mast cells and plays a role in fibrosis in HL. IL-9R expression is found on cells resembling mast cells in HL which also supports an interaction with these cells.

Despite a clear correlation between IL-9 and the number of eosinophils and mast cells (both of which have prognostic importance in HL [78, 85, 86]), and the possible autocrine stimulation of the HRS cells [100], no influence of IL-9 on survival in HL was detected. However, patients with IL-9-positive HL tumours had higher WBC and ESR, both parameters related to inflammation and poor prognosis. High levels of IL-9 in serum also correlate to negative prognostic factors such as high ESR, high WBC, B-symptoms, and advanced stage disease [102]. In a study investigating 30 different cytokines, IL-9 in sera did not show any association to early treatment failure; however the correlations to negative prognostic factors is not reported [103]. In that study the cytokine IL-10 was the only cytokine to be an independent predictor of early treatment failure.

The lack of correlation to poor prognosis among the patients with IL-9-positive tumours was a surprising result. However, the material consisted of 131 patients all of whom have good prognosis with modern treatment. There was a slight overrepresentation of young patients among those who were positive for IL-9. Younger patients tolerate intensive treatment better than older patients do, which made the results difficult to interpret. IL-9 is probably involved in the pathogenesis of HL, but with modern treatment its role as a prognostic marker appears minimal.

From a tumour biology perspective IL-9 expression, both in the tumours and in sera, correlates to NS histology. This indicates that IL-9 and its receptor, among other cytokines [27, 102, 146], are involved in fibroblast activation, leading to the dense fibrosis seen in these tumours.
III Angiogenesis and mast cells in Hodgkin lymphoma.

In Paper III, HL patients with a high MVC, cut off at the 75th percentile (n=33), had a worse PFS than to those with a lower number of microvessels had (Figure 10). The HLS was not significantly influenced by the number of vessels (p=0.17). When tested by multivariate analysis against age and low s-albumin, the most powerful prognostic factors, microvessel count was not statistically significantly related to PFS (p=0.10), probably due to the strong influence of age on prognosis.

![Figure 10](image)

*Figure 10.* Patients with high microvessel count have a worse PFS than patients with low microvessel count.

These results agreed with another report [114] indicating a high MVD predicted poor prognosis in HL. Like us, they noted a positive correlation with high age and microvessel density (MVD). This should be considered when evaluating the results. In another study [147] MVD increased with disease progression in 7/11 cases of classical HL.

One unexpected result was that a high MVC did not correlate to a high number of mast cells (Spearman R=0.05, p=0.62) in our study, as it does for other lymphomas [109]. This might indicate a different pathway for the proliferation of microvessels in HL than in other lymphomas.

VEGF, a major angiogenic molecule, is produced by HRS cells in HL [111]. Eotaxin, expressed in HL [148], also induces the formation of blood vessels, and consequently, may contribute to vascularisation. Reactive
macrophages in HL are also positive for VEGF, indicating that they contribute to angiogenesis [111]. Thus, several possible angiogenic factors and ways of stimulating angiogenesis exist in HL.

A contribution of mast cells to microvessel stimulation cannot be totally excluded, but it was not reflected in mast cell numbers. Mast cells express several angiogenic factors, and variations in production and release of these, or other related compounds, may be of greater relevance than the mast cell numbers are. As most leukocytes, including mast cells, produce a myriad of angiogenic factors, it is likely that they modulate the formation of new vessels in HL. These cells also generate angiogenesis inhibitors, so their overall role in initiating or terminating angiogenesis depends on the temporal and spatial balance of these molecules [106]. This needs to be separately evaluated for each cell type, each factor, and each disease.

In conclusion, this study adds further evidence that high MVC is of importance for HL prognosis, yet this must be evaluated in larger materials where age as a confounding factor must be taken into greater consideration. To be able to cure the few but existing patients that are resistant to therapy, new treatment concepts, such as angiogenesis inhibitors must be explored [149]. The lack of correlation between high mast cell count and high MVC can lead in other directions when studying the role of mast cells in HL.
The eosinophil infiltration characteristic of many HL-affected lymph nodes is different from reactive lymph nodes, where almost no eosinophils are present (unpublished data). Eosinophils stained specifically for ECP were present around HRS cells (Figure 11), and a majority (60%) of HL patients have elevated ECP in serum (S-ECP) [93].

The presence of eosinophils are alleged to stimulate tumour progression and eosinophilia is a negative prognostic factor in some studies [85, 86]. Despite this, ECP was cytotoxic to the HRS cells from the HL cell lines HDLM-2, KM-H2 and L428 in vitro. The cytotoxic profile of ECP was similar to a carefully investigated cell line (H69) (Figure 12). ECP was cytotoxic at very low concentrations and most pronounced for the HDLM-2 cell line, of T-cell origin. The effect did not increase proportionally at high concentrations, and in the HDLM-2 cell line, a plateau was actually reached (Figure 12). A substantial fraction remained alive even at high concentrations and a prolonged exposure time (up to 72 hours). When HRS cells from the HDLM-2 cell line were put back into culture after ECP exposure, they continued to grow. The different genotypes and level of glycosylation did
not affect cytotoxicity on HDLM-2, in contrast to H69 (a small-cell lung cancer cell-line), indicating that the mechanism causing cytotoxicity on HDLM-2 was different from that on H69.

**Figure 12.** Cytotoxic effect of ECP on HDLM-2 (green line) compared to L428 (blue line), KM-H2 (red line) and H69 (purple line). ECP was added in increasing concentrations and incubated for 72h before evaluation. The results are given as mean survival index (SI%) of two experiments (in duplicate). The ECP fraction tested is an ECP97arg protein with a low level of glycosylation.

In conclusion, the plateau effect of the cytotoxic properties of ECP on HDLM-2 may explain why many eosinophils can be present around the tumour cells, but not totally eradicate them. Whether the cell lines are derived from patients with eosinophil rich tumours, or tumours with few eosinophils is unknown, but could affect the resistance to ECP identified. ECP may help in the selection of more resistant HRS cells.

Tumour cells in eosinophil-rich HL cases are less likely to be EBV positive (Paper V), more frequently secrete IL-9 and express IL-9R (Paper III), contributing to a stimulatory loop for the HRS cells and they, as other HRS cells, express CD30 and are not CD20 positive to a greater extent than HRS cells in non-eosinophil-rich cases (data not shown). They are perhaps more sufficient for further survival than tumour cells in non-eosinophil rich cases; however, this is not proven but an interesting hypothesis.
V Predictors for histology, tissue eosinophilia and mast cell infiltration in Hodgkin Lymphoma – a population-based study

In a population based study of 448 patients, the SCALE material, interview data about possible aethiological factors for HL were related to tumour tissue characteristics. For the first time, the possible contribution of host-related factors to the composition of the inflammatory infiltrate, especially eosinophilia in HL was demonstrated. Factors related to tissue remodelling; asthma, and having the polymorpism ECP434GG increased eosinophil numbers in the tumours as did EBV infected tumour cells, and being a smoker lowered the numbers. Factors related to high eosinophil count were associated with activated and primed eosinophils (asthma, and possibly hives, and autoimmunity), whereas, factors associated with lower numbers were related to a suppressed immune system, smoking, and having an EBV infection.

People presenting with certain histology have certain characteristics, such as patients with NSHL are usually younger, more often have EBV negative tumour cells, and are more often female. In this study, an attempt was made to determine if patient traits affected the histology of the HL tumour. However, only known factors such as younger age, female gender, and EBV negativity predicted the histology of NSHL, with EBV status being of importance in multivariate analysis. Other diseases, such as asthma or autoimmunity did not influence histology. As host-related factors affect eosinophilia, which is part of the definition of the different histologies, they may still affect the histology in the individual setting, but this was not shown in this large material and the effect is probably marginal.

The factors of strongest predictive value in multivariate analysis for high eosinophil count was the ECP genotype. An individual’s genotype in combination with the characteristics of the transformed HRS cell may affect the histology a person presents with.

A difference from previous studies between the associations of ECP genotypes and allergic symptoms was seen. Individuals with ECP434CC more often reported hayfever and were more often atopy positive than individuals with ECP434GG. This concurred with the immunological derangements in individuals with HL and indicated that a persons genetic composition can affect the raised IgE levels from the HL disease differently and also affect the symptoms of allergy differently.

When investigating tumour microenvironment in different tumours and its relation to prognosis, both the properties of the tumour cells and the traits of the patient could affect the results and need to be considered. This does not diminish the value of a prognostic factor. However, it is important to understand why a certain factor is prognostic in order to make the correct
conclusions. This raises the question as to whether this information can provide a hypothesis for the cause of EBV negative HL.

The factor that drives the disease often constitutes the best therapeutic target. In the future, it would be beneficial to be able to take an ordinary medical history, and by this define what patients to investigate for specific treatment predictive factors, such as histopathological characteristics, typical mutations, and/or genetic characteristics. This could then help to decide the targeted therapy from which the person would benefit the most.
Summary of results

I Patients with stage IIB HL, diagnosed and treated in Sweden between 1985 and 1994, had a prognosis that was equivalent to patients with stage IV disease. Bulky disease in the mediastinum predicted patients with poor prognosis the best.

II IL-9 expression from HRS cells is related to high number of tumour eosinophils, mast cells, the histology of nodular sclerosis, and to negative prognostic factors.

III A high microvessel count was related to a poorer disease-free survival and to high age; however, it was not related to high number of mast cells in tumours.

IV Eosinophil protein ECP was cytotoxic to HL cell lines at low concentrations: at high ECP concentration, the cytotoxic activity was more pronounced for the two cell lines of B-cell origin than for the cell line of T-cell origin.

V Patient traits, such as having had asthma, hives, and having the genotype ECP434GG, were associated with a higher infiltrate of eosinophils in the tumour than not having these traits. Smokers and patients with EBV-positive tumour cells had fewer eosinophils than non-smokers and patients with EBV negative tumours.
Figure 13. Summary of the results: Hodgkin and Reed-Sternberg cells, eosinophils, and mast cells interact, which affects fibrosis and angiogenesis in HL tumours. The patient’s traits also affect these interactions by partly deciding the number of infiltrating eosinophils in the tumours.
General discussion and future studies

General perspective of prognostic and predictive factors
In patients with HL, there is an urgent need to determine factors that can predict prognosis at the time of diagnosis. Although several studies have addressed this, it is still uncertain which patients will experience a relapse or which patient has a primary progressive disease after a particular standard therapy. Prognostic indexes, such as the IPS, are currently the most applied way to predict prognosis and stratify patients to different treatments. Other clinical characteristics, such as bulky disease, are also good prognostic markers. In addition, the results from a PET scan after two courses of CT (and possibly already after one course) appear to be a good marker, and this is now evaluated in several clinical trials.

Prognostic markers change when therapy changes and targeted therapy allows a move away from the conventional view of negative prognostic factors towards relying instead on molecular and genetic markers to predict which targeted therapy to use. One future aim in the area of targeted therapies, is to be able to predict the efficacy of e.g. anti-angiogenic treatments through a property reflecting vascularity/neo-angiogenesis; another option can be used in eosinophil rich cases, and fibrosis-rich tumours could predict therapy in a third way.

However, it is difficult, time consuming, and costly to investigate patients for specific predictive factors. Knowledge about how simple clinical parameters (such as asthma, hives and smoking status) or laboratory tests correlate to specific predictive markers can help in the selection of patients to investigate for these predictive markers. One possibility is to take an ordinary medical history and identify patients more likely to present with a specific target, that indicates high or very low probabilities to respond to a specific drug. Even if a predictive test is cumbersome and expensive, it may save costs and toxicity, as newly developed drugs are costly.

Identify how personal traits affect HL presentation at diagnosis
With the help of epidemiological information, such as collected in the SCALE material (Paper V) and clinical information about the patients, it may be possible to identify which personal traits affect how the patients present at the time of initial diagnosis. One study already planned is to investigate if, for example, patients with autoimmune diseases have a higher
or lower ESR, early or more advanced stage, and a worse or better prognosis. Several other traits and co-morbidities, such as for example asthma, smoking, and hayfever (as presented in Paper V) will be investigated to determine how they affect clinical characteristics and prognosis. This would benefit the clinician in an everyday work, as it is difficult to evaluate patients with co-morbidity, and will be of importance for both risk factor judgment and for individualising treatment decision.

A map of a ‘nice’ and a ‘hostile’ microenvironment
One ongoing investigation on the SCALE material is the evaluation of the number of macrophages, the number of regulatory T-cells, and CD20 expression on the HRS cells, in addition to the number of eosinophils and mast cells already explored. There is an overlap of cases with many mast cells and many eosinophils, but how the other cell types are linked to each other is unknown. From the pathologist point of view, can a ‘nice’ or a ‘hostile’ microenvironment be identified?

A further contribution to this knowledge is to elucidate the determinants of the composition of the microenvironment, through the epidemiological, clinical, and tumour material available in the SCALE material, and to determine if host traits affect the infiltration of cell types other than eosinophils and mast cells. This would contribute to individual risk assessment, prevention, and possible future directions for targeted therapy.

General perspective of future therapy
The conventional drugs available need to be optimised and used in the best combinations. For patients with early stages of disease, the high cure rates need to be maintained while reducing late toxicity. For intermediate stages, individualised treatment is needed, some patients require reduced treatment, and others require increased treatment. For the advanced stages, cure rates need improving, and a combination of conventional drugs with new drugs for the few but still existing patients that do not respond properly to treatment or relapse is possible. Many new targeted-dugs are available for use in human malignancies, including HL.

The microenvironment and drug resistance
The role of the microenvironment in drug resistance is not fully appreciated. To reach all tumour cells, drugs must be delivered efficiently through the tumour vasculature, cross the vessel walls, and traverse the tumour tissue [150]. Heterogeneity in the microenvironment may cause different regions of hypoxia and acidity, all of which can influence the sensitivity of the tumour cells to drugs and radiation treatment. Agents that improve drug delivery or activity by targeting the microenvironment represent an important future
direction for cancer therapy. For example, is it possible to inject a dye into the vessel providing blood to the node, before an enlarged lymph nodes is removed, and investigate the areas reached by the dye after lymph node removal? Can the characteristics of tumour cells to target, which are far away from the dye, be identified? Is it possible to identify cells that protect the tumour cells, and target those? Is the reduced activity on FDG-PET after two courses of CT attributed to the killing of the tumour cells so that the microenvironment can no longer metabolise and reside in the tissue? Or is the reduced activity on FDG-PET attributed to the killing of the cells in the microenvironment, so that the tumour cells eventually cannot survive without its “tissue support”?

New PET tracers and new magnetic resonance (MR) sequences may help in this improved characterisation of the tumours before and during treatment. Our group in Uppsala have planned for such studies.

Is it possible to label HRS cells, with for example antibodies, which could potentiate the effect of ECP?

Better models for investigating the effects of eosinophils or other immune cells on HRS cells could be developed by co-culturing experiments of eosinophils, HRS cells, macrophages, and/or fibroblasts. Three-dimensional cultures, as described in [151], would possibly present an opportunity to investigate the microenvironments influence on HRS cells.

A therapeutic approach would be to identify concepts that trigger the immune system to kill the tumour cells. In vitro data indicate that antibodies partly, and presumably, work by binding to the tumour cells, and cell death is then mediated via antigen dependent cellular cytotoxicity (ADCC). The importance of ADCC in the patient is conflicting and under investigation [152]. ECP is cytotoxic to some, but not all, HL cell lines, and not sufficiently. Labelling of resistant tumour cells would possibly make them easier targets for ECP? Unspecific binding of CD20 to the cells exposed to ECP was identified in this study (in preliminary flow cytometry experiments in Paper IV). The cells may have been damaged by the innate immune system, thus, becoming more sensitive to unspecific binding of monoclonal antibodies than non-damaged cells. One new approach would be to trigger the immune system to be more effective in killing tumour cells, and then, add therapy with e.g. monoclonal antibodies.

Ibland drabbas vita blodkroppar av mutationer, genetiska förändringar, som kan leda till att de börjar växa okontrollerat samt bli odödliga. I vissa fall kan en klon av identiska tumörceller bildas vilket orsakar en livshotande cancersjukdom. När det drabbar vita blodkroppar i lymfsystemet kallas det lymfom (lymfkörtelcancer). Eftersom det finns en mängd olika typer av vita blodkroppar som kan befinna sig i olika stadier av utveckling kan vi drabbas av många varianter av lymfom.

Den här avhandlingen handlar om den lymfomvariant som kallas för Hodgkins lymfom (HL) (tidigare benämnd Hodgkins sjukdom). HL härstammar i de flesta fall från B-lymfocyter och i sällsynta fall från T-lymfocyter som är en annan typ av vita blodkroppar. HL kan drabba både barn, unga vuxna och gamla människor. Man vet idag att en infektion av Epstein-Barr Virus (EBV), samma virus som orsakar körtelfeber tidigare i livet, hos vissa individer är en bidragande orsak till sjukdomen. EBV finns då kvar i den B-lymfocyt som tagit hand om viruspartikeln och detta bidrar på sikt till att just den här B-lymfocytten blir odödlig, vilket kännetecknar en cancercell. Hos de flesta som drabbas av HL vet man dock fortfarande inte vad orsaken till sjukdomen är.

HL yttrar sig som förstorade lymfkörtlar, oftast på halsen eller i området mellan lungorna. Vissa drabbas också av besvär som viktnedgång, feber eller nattsvettnings, så kallade B-symptom. Besvären orsakas av de ämnen som utsöndras från tumöreerna. Med cellgifter och strålbehandling, som ges i olika mängd beroende på sjukdomsutbredning, så botas idag de allra flesta som drabbas av HL. Behandlingen är nödvändig för att bota sjukdomen men patienten riskerar att av denna drabbas av andra tumörer, hjärt- och lungsjuksjukdomar eller andra allvarliga biverkningar på sikt. För att kunna behandla sjukdomen effektivt med minimala biverkningar är det av största
vikt att veta hur spridd sjukdomen är. Helst vill man kunna förutspå hur det kommer att gå för den enskilda patienten, för att kunna ge en individanpassad behandling. En idé kan därför vara att hitta faktorer som förutsäger prognosen redan vid diagnostillsfallet, så kallade prognostiska faktorer.


Vad är då dilemmat som finns idag vid HL och vilka frågor har vi försökt svara på? Målet är att försöka bota alla som drabbas, med så lite behandling som möjligt för att undvika de allvarliga biverkningarna som behandlingen ger. Ett annat mål är att bota de få patienter där nuvarande behandling inte fungerar. Vi vill också förstå hur mikromiljön uppkommer i tumörerna och vad den har för betydelse, så att nya läkemedel med mindre allvarliga biverkningar kan utvecklas.

**Delarbete I**


Om man jämför med de patienter som har mer spridd sjukdom går det förhållandevis dåligt för patienter med stadium IIB, trots den begränsade sjukdomsutbredningen. De patienter som hade >10cm stor tumör, i området mellan lungorna hade sämst prognos av alla. Dessa patienter bör få intensivare behandling än vid tidigaregett, för att vi ska ha en chans att bota dem. Efter denna studie rekommenderas det för den här patientgruppen i Sverige idag en mer intensiv behandling än tidigare.
Delarbete II
I tumörmaterial från 130 patienter med HL från alla stadier, har jag relaterat fynd i tumörerna till klinisk information.

Cytokinen IL-9 har tidigare visat sig stimulera HL tumörcellerna i odling till ökad tillväxt. Vi har nu visat att IL-9 uttrycks av tumörcellerna hos cirka hälften av patienterna. De patienter med IL-9 positiva tumörceller hade fler antal eosinofiler och mastceller i sina tumörer. Dessa patienters tumörer var också mer bindvävsrika än andra patienters tumörer och patienterna hade högre sänka vid diagnos och fler vita blodkroppar i blodet, vilka är negativa prognostiska faktorer.

Delarbete III
I tumörerna jag undersökte i arbete två har jag gått vidare och räknat antalet blodkärl och relaterat detta till klinisk information.


Delarbete IV
Här har jag i cellodlingsförsök studerat hur ett ämne som utsöndras av de eosinofila granulocyterna (eosinofilt cationic protein=ECP) påverkar tumörcellerna.

Eosinofilerna tros stimulera tumörcellerna till ökad tillväxt men ECP i sig är känt som ett celldödande protein, vilket för oss var en paradox. Vi visade att ECP trots allt kan döda tumörceller vid HL. För en cellinje, (representerande den ovanliga varianten av HL som kommer från T-lymfocyter), klarade ECP inte av att döda HRS cellerna i samma utsträckning som de två cellinjer som kom från B-lymfocyterna. Antalet undersökta cellinjer är dock bara tre, varför det inte går att dra säkra slutsatser.

Delarbete V
Här försöker jag förklara vilka egenskaper hos patienterna som spelar roll för hur många eosinofiler och mastceller man får i sin tumör. Detta har vi undersökt hos alla som insjuknat med HL i Sverige eller Danmark mellan
åren 1999–2002 och som accepterade att vara med i studien och där tumörmaterial fanns bevarat i tillräcklig mängd, (448 patienter).

De patienter som har haft astma, nässelfeber, någon autoimmunsjukdom eller som har en specifik genetisk variant av ECP har fler eosinofiler i sina tumörer. De patienter som tidigare i livet har rökt eller vars tumörceller var infekterade med EBV-virus hade tendens till färre eosinofiler. Detta är helt ny kunskap och av betydelse för förståelsen av vad som bestämmer hur mikromiljön i en individs tumör ska se ut.

Sedan tidigare vet man att personer med autoimmuna sjukdomar har lite ökad risk att drabbas av lymfom jämfört med andra människor, att rökare har ökad risk att drabbas av EBV-positiv HL och vissa studier tyder på att de med allergisk sjukdom har något minskad risk att drabbas av HL, medan andra studier inte har visat någon korrelation. Hur dessa fynd hänger ihop med mikromiljön i tumörerna är fortfarande okänt. Eventuellt kan dock den inflammation (med många eosinofiler) som autoimmunitet, astma och nässelfeber orsakar vara med och förklara utvecklingen av EBV-negativ HL. Prognosen för HL har successivt förbättrats i Sverige sedan slutet av 60-talet vilket visats i en publikation av M Talbäck 2003 (Figure 14), en ytterligare förbättring är troligtvis något vi kan vänta oss framöver.

HRS-celler kan inte överleva som enda cell i en HL tumör. Istället är tumörerna en komplext vävnad som består av ditrekryterade och påverkade normala celler, bindväv och blodkärl, vilka troligtvis stödjer HRS-cellerna. I framtiden kommer vi kanske att skräddarsy behandling utifrån vilka levnadsvanor och sjukdomar patienterna har haft tidigare i livet och genom att specifikt titta på utseendet av individens mikromiljö runt tumörcellerna.

![Figure 14. Relativ överlevnad för Hodgkin Lymfom patienter i Sverige, vänster bild visar män 0-89 år och höger bild kvinnor 0-89 år. Hämtad från M Talbäck Acta Oncol 2003 42(7). Publicerad med tillstånd från Informa.](image-url)
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