Regulatory T cells in Hodgkin’s Lymphoma

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Abstract

Background and purpose: Recent studies on Hodgkin’s Lymphoma (HL) have investigated the prognostic significance on the amount of regulatory T cells (Treg) in tumour sections. In earlier studies there has been a shorter overall and disease free survival in the group of patients having few Treg, in some studies however contradicting results have been found. Supposedly Treg interacts in the antitumoral host immune response. The purpose with this study is to evaluate the amount of Treg in tumour sections and its relevance for the prognosis, tumourbiology, and clinical characteristics in this group of patients.

Patients and methods: 323 previously prepared tumour sections were counted by the author on whole tumour sections in 40x magnification, calculating the percentage of Treg in relation to the total amount of lymphocytes. 121 cases had previously been counted by an experienced hematopathologist on Tissue Micro Array (TMA) slides. Antibody used was anti-FoxP3 MoAbs using immunohistochemistry.

Results: High amount of Treg cells affects prognosis negative with a higher amount of patients deceased or relapsing following the disease. In the group of patients above 45 years of age, patients with >15% Treg cells in their tumours had a shorter disease free survival (p=0.03). High number of Treg cells correlated with eosinophilia and positive EBV status, as well as a young age.

Conclusions: In contradiction to previous studies we here see that many Treg cells is associated with shorter disease free survival, especially in older patients. In accordance with other studies it would be interesting to evaluate the amount of Cytotoxic T Lymphocytes (CTL) on the same material. It is also needed to collect survival data from Danish patients whose tumour sections were counted in this study to see if the results remain the same within a larger cohort.

Background

Hodgkin’s Lymphoma (HL) is a relatively rare malignancy with good short-time prognosis. A majority of patients are young adults that in order to be cured are treated with chemotherapy and radiotherapy. Treatment of the disease often results in secondary complications such as malignancy and cardiopulmonary disease. The goal is to provide enough treatment so the patients are cured, without giving too much in order to avoid complications. This is still a challenge in the treatment of lymphomas. Deciding the amount of treatment is based on prognostic factors; finding of new prognostic factors is therefore demanded. It is of great interest to further investigate the interesting biology of this disease where only a small fraction of cells in affected tumour-tissue are malignant Hodgkin Reed-Sternberg (HRS) cells while other cell-types make up a complex network of signalling cells. Knowledge about the biology gives opportunities to find new prognostic factors, possibly predicting what treatment to use and also give opportunities to develop new less toxic drugs. Several different cell-types in the environment surrounding HRS cells have previously been shown to be significant in disease prognosis, including eosinophils, mast cells and cytotoxic
CD8+ T cells. Epstein-Barr virus (EBV) is present in HRS cells in about one third of HL cases, but its exact role remains uncertain.

The most common infiltrating cell in the tumour tissue is CD4+ T cells and a fraction of these are regulatory T cells (Treg). Treg cells have a suppressing function on immune-response cells by inhibiting cytotoxic CD8+ T cells and Natural Killer (NK) cells, which role is (among others) to kill neoplastic cells. In theory it would therefore be an advantage with few Treg cells, resulting in high numbers of CD8+ T cells and effective eradication of neoplastic cells. Recent studies have indicated the opposite to be correct, high number of Treg cells and low cytotoxic T cell count has been accompanied by a favourable prognosis [11-13, 16]. However contradicting results have also been seen [14]. The exact role of the number of Treg cells as a prognostic marker remains thus still unknown.

**Aim**

The aim of this study was to investigate the clinical and biological importance of Treg cells in HL in a large patient material. The aim was to see if the number of Treg cells can be used as a prognostic factor and to investigate its relation to EBV status and other inflammatory cells.

**Specific research questions**

- A high infiltration of Treg in HL have in several studies been identified as a good prognostic marker [11-13, 16], however when the ratio of Th2 cells and Treg cells was utilized contradicting results have also been seen [14]. How does the number of Treg cells in this large population based material relate to prognosis and to other clinical parameters in HL patients?
- One previous study did not show any correlation between EBV status and the number of Treg cells [11]. However experimental studies have shown that EBV positive HL tumour cells express the chemokine CCL20 that cause Treg cells to migrate towards the tumour cells [5, 15]. We want to investigate how the number of Treg cells in this material relates to EBV status.
- T cells in HL, and among them Treg cells have been shown to be anergic (not functioning as proper immune cells) [17]. Experimental studies of the interactions between CD4 positive cells and eosinophils have shown that when the CD4 positive T cells are in an anergic state the eosinophils cannot mediate their normal functions [18]. In studies of HIV patients with total lack of CD4 positive cells no eosinophils are detected either. What is the relation between the number of eosinophils and the number of Treg cells in HL tissue?
- Activated Treg cells are also known to recruit and activate mast cells to mediate a regional immune suppression [19]. How the relation between the number of mast cells and the number of Treg cells in HL is unknown, especially since the Treg cells in HL possibly are in an anergic state.

**Introduction Hodgkin’s Lymphoma**

**History**

Hodgkin’s Lymphoma (HL) was first described by Thomas Hodgkin in 1832 in a paper entitled “On Some Morbid Appearances of the Absorbent Glands and Spleen” published in *Medical-Chirurgical Society Transactions*, he described seven patients having enlarged lymph nodes, not associated with pain, heat or due to metastasis of
adjacent tumours. Subsequent histopathological examinations revealed that three of the seven cases were HL. HL has also been known as Hodgkin’s disease which was first coined by Samuel Wilks in 1865. Today, Hodgkin’s Lymphoma is the most appropriate and commonly used name for the disease. [1]

**Pathology**

HL is a monoclonal lymphoid neoplasm, derived from B cells, composed of mononuclear Hodgkin cells and multinucleated HRS cells residing in an abundant cellular microenvironment. Microenvironmental cell types include small non-neoplastic B and T lymphocytes, plasma cells, eosinophils, mast cells, histiocytes, and macrophages. Interplay between different cells in the microenvironment via cytokines and chemokines results in progression of the lymphoma. [3]

HL arise almost invariably in a single lymph node and spreads to anatomically contiguous lymph nodes. Primary extranodal HL is uncommon and is often associated with HIV-infection. Extranodal engagement is common as a secondary phenomenon from a primary lymph node. Common extranodal sites are spleen, bone marrow, liver, thymus and lung. Unusual but reported sites of involvement are gastrointestinal tract, tonsillar tissue and skin. [1]

**Morphology**

Carl Sternberg in 1898 and Dorothy Reed in 1902 published independently a description of the cytologic features of the neoplastic multinucleated giant cells, which have since been known as Reed-Sternberg (RS) cells. Mononuclear RS cell variants are designated as Hodgkin cells, all variants of neoplastic cells in HL are referred to as Hodgkin Reed-Sternberg (HRS) cells.

Reed-Sternberg (RS) cells are large (15-45µm in diameter), with a multilobated nucleus, each lobe contains one large inclusion-like eosinophilic nucleus (size up to 10µm). Chromatin surrounding the nucleus often shows a clear zone. Particularly characteristic are RS cells with an owl-eye appearance; being two mirror-image nuclei or nuclear lobes with acidophilic nucleolus surrounded by a clear zone.

Lacunar cells is a mononuclear RS cell variant; it has a single multilobated nucleus, multiple small nucleoli and abundant pale-staining cytoplasm.

In 1966 Lukes and Butler described a multilobated variant of the HRS cells, the L&H cells. L&H (often referred to as “popcorn cells”) cells are large and have lobulated nuclei and small to moderate-sized basophilic nucleoli, occasionally resembling lacunar cells. [1]

**Subtypes of HL**

HL consists of two different clinical, pathologic and biologic disease entities; classical Hodgkin Lymphoma (cHL) and Nodular Lymphocyte Predominant Hodgkin Lymphoma (NLPHL). CHL is further divided into Nodular Sclerosis (NS), Mixed Cellularity (MC), Lymphocyte Rich (LR) and Lymphocyte Depleted (LD). Material for this study includes only cases with cHL, so therefore this entity is described in more detail. NLPHL subtype will not be further described in this paper.

NS is by far the most common type of cHL accounting for more than two-thirds of the cases. It is characterized by lacunar cells and presence of one or more sclerotic bands, which usually radiate from a thickened lymph node capsule, often following the course of a penetrating artery. Lacunar cells are the most common type of HRS cell present and can often be found in large numbers. Diagnostic typical RS
cells can usually not be identified. Eosinophils and neutrophils are often numerous. Histiocytes and plasma cells are sparse.

MC compromises about 30% of cHL cases in the western world, but may be more abundant in developing countries. A heterogenous mix of Hodgkin cells, small lymphocytes, eosinophils, neutrophils, histiocytes, plasma cells, and fibroblasts are present. Typical RS cells and mononuclear variants are abundant and easy to find. Small foci of necrosis may be present, but to a far lesser extent than seen in NS.

LD encompasses two types of cHL; diffuse fibrosis and reticular. Diffuse fibrosis is characterized by a marked degree of fibrosis surrounding single cells along with lymphocyte depletion. Fibrosis does not consist of thick bands as in NS. Hodgkin cells are usually easy to identify. In the reticular variant sheets of Hodgkin cells showing pleomorphic features are found along with lymphocyte depletion. The LD subtype is today rare and historically many cases originally classified as LD have been reclassified as cases from diffuse large B cell lymphomas.

In LR subtype HRS cells are usually rare and have identical features to cells in the MC subtype. The background is dominated by numerous small mature lymphocytes. Eosinophils and neutrophils are often restricted to blood vessels. Some variants may resemble NLPHL subtype. [1]

**Immunohistochemical investigation**

HRS cells have lost the typical B cell surface and functional phenotype and display an abnormal repertoire of membrane markers. [3] Immunohistochemical studies with paraffin sections are of great use in the diagnosis of HL. A limited panel of monoclonal antibodies provide useful information using Cluster of Differentiation 45 (CD45), CD40, CD 15, CD30 and CD20. CD45 can be used as a “negative” marker for HL, only expressed by 7% of HL cases, while expressed by 97% in B-cell lymphoma. [1] cHL HRS cells consistently express CD30 and CD40, CD15 in about 80% of cases, CD20 in about 35% of cases. [3]

L&H cells have a typical immunohistochemical profile, positive for CD45, CD20, and negative for CD30 and CD15. [1]

**Cellular microenvironment**

In summary, the cellular microenvironment in cHL plays a crucial role in allowing the HRS cells to survive, by providing an environment that suppresses cytotoxic immune responses and effective tumour-killing. Future therapeutic strategies could act by inhibiting the receptors to ligand interactions, the chemokine and cytokine network, or the induction of effective anti-EBV latent membrane protein immune responses. [3]

**HRS cells**

Knowledge about the molecular mechanisms leading to HL is poor, mainly due to the poor distribution of neoplastic HRS cells in tumour mass where they represent less than 10% of total cell population in the lymphoma. This makes cytogenetic and molecular analyses challenging, which resulted in long-lasting uncertainty of the cells true origin. [1]

The origin of HRS cells was clarified when microdissection and single-cell PCR methods were applied to single HRS cells to analyze them for immunoglobulin and TCR gene rearrangements. Nearly all cases carry mutated immunoglobulin variable gene rearrangements. Such mutations take place in antigen-activated B-cells participating in immune responses in germinal centers through the process of somatic mutation.

Regulatory T cells in Hodgkin’s Lymphoma
Regulatory T cells in Hodgkin’s Lymphoma

hypermutation. Therefore HRS cells are derived from germinal-center or post-germinal center B-cells. Normally B-cells with destructive somatic mutations undergo apoptosis within the germinal centers and are removed by macrophages. There is only a few cases of cHL where they lack rearranged V-genes, probably from pre-germinal center naive B-cells. [1]

L&H cells lack crippling mutations and show intraclonal variable gene diversity, indicating hypermutation activity during clonal expansion, they hence appear to derive from selected, mutating germinal-center B-cells. Active hypermutation is a hallmark of germinal-center B-cells and indicate that L&H cells are of germinal-center B-cell origin. Rearrangements of the TCR genes have also been found, indicating HRS in rare cases originates from T-cells (less than 2%). [1]

HRS cells are dependent of CD40 ligand (CD40L) expressing T cells, CD30 ligand (CD30L) expressing mast cells, eosinophils and non-neoplastic B cells for their survival. [3]

The NF-κB transcription factor is a hallmark in HRS cell proliferation and regulates the expression of multiple anti-apoptotic factors and pro-inflammatory cytokines and plays a crucial role in the pathogenesis of cHL. HRS cells express CD40 and both Receptor Activator for Nuclear Factor κ B Ligand (RANKL) and its receptors RANK, and activation of RANK and CD40 contributes to NF-κB activation and induction of several cytokines, such as IL-6, IL-8, CCL5, IFNγ and IL-13, thus contributing to the maintenance of an inflammatory environment in cHL. [3]

The constitutive activity of NF-kB is also caused by genetic abnormalities. The TNFα-induced protein 3 (TNFAIP3) gene encodes A20, a negative regulator of the NF-κB pathway, genetic studies have recently revealed that A20 is frequently inactivated in several B-cell lymphomas including HL. [3]

Figure 1: Classic Reed-Sternberg cell with characteristic owl-eyes.

**T-Lymphocytes**

Lymphocytes in close vicinity to HRS cells are almost always exclusively positive for CD4+ and are of the T helper (Th) and Treg subtype, usually very few are CD8+ cytotoxic T cells or natural killer (NK) cells in this area. Th1 cells support inflammatory responses by producing IL-2 and IFN-γ, Th2 cells support humoral responses by producing cytokines such as IL-4, IL-5 and IL-10. Patients have elevated serum levels of Th2 cytokines and frequently have elevated levels of IgG and IgE in HL, consistent with the proposal of Th2 being the main Th subtype. [1]

Regulatory T cells in Hodgkin’s Lymphoma
Regulatory T cells
Regulatory T cells are a type of T-lymphocyte; it is also known as CD4+CD25+FoxP3+ Regulatory T cells and commonly referred to as Treg cells. They function as a suppressor of immune response (formerly known as suppressor T cells) via several mechanisms. Treg cells express the transcription factor and surface protein forkhead box P3 (FoxP3) which is mandatory for this T cell phenotype, it is considered to be the most specific marker for Treg to this date. [2] FoxP3 is used as a specific marker of Treg cells in this study.

Treg development
T cells originate in bone marrow from multipotent lymphoid stem cells and migrate to the thymus for further differentiation and proliferation. Thymic development of Treg cells requires high-affinity interactions between their T cell receptor (TCR) and self-peptide-MHC complexes presented by thymic stromal cells, these cells also provide co-stimulatory signals necessary for Treg cell development. Both IL-2 and IL-7 in thymic milieu are required for development of Treg in mice. Thymic Stromal lymphopoietin (TSLP) secreted by Hassall’s corpuscle in thymus activate immature CD11c+ Dendritic Cells (DCs) and upregulates their expression of co-stimulatory molecules. These DCs induce FoxP3 expression in immature CD4+CD8-CD25-thymocytes and support differentiation into mature FoxP3+Treg cells. Sustained FoxP3 expression by activated T cells is required for Treg cells suppressive function. [6]

CD45RA expression without concomitant expression of CD45RO is a phenotypic marker for naive T cells that have not experienced TCR stimulation-mediated maturation. They also express low levels of intracellular FoxP3 and lack expression of Ki67, a nuclear proliferation marker, indicating their quiescent state. During activation, naive Treg cells proliferate, upregulate FoxP3 and convert to CD45RO+CD25+FoxP3+Treg cells. [6]

Up to this date, several types of Treg cells have been described and they can arise both in the thymus and in the periphery. Natural regulatory T cells (nTregs) with CD4+CD25+FoxP3+ phenotype are generated in the thymus, different from the other subtype known as induced regulatory T cells (iTregs), which are induced following MHC-peptides stimulation, or by cytokine-exposure in peripheral tissue. Different subsets of iTregs includes CD4+ iTregs, CD8+ iTregs, Tr1, and Th3. Recently it was discovered that nTregs express Helios (a zinc finger protein) in a higher extent than iTreg, which may be used as a marker to differ between these two subtypes of Treg. [5, 24]

Treg function
Treg suppress cytotoxic CD8+ T cells via several mechanisms, including modulation of cytokine microenvironment, metabolic disruption of target cell, alteration of Dendritic Cells (DCs) activating capacity and cytolysis. Cytotoxic T Lymphocyte Antigen 4 (CTLA4) expressed by Treg can modulate CD80 and CD86 expression by DCs and thereby inhibit the activation of cytotoxic CD8+ T cells. [6]

Tregs chief mechanism in inhibiting the function of cytotoxic CD8+ T cells and NK cells is by cell-to-cell contact via Perforin/Granzyme B pathway or cytokine-dependent by IL-10 or TGF-ß resulting in apoptosis of affected cells. Tumours can recruit circulating nTregs from peripheral blood by expressing chemokines CXCL12 and CCL22, or induce the production of Tr1 cells from CD4+ naive T cells by cytokines such as Cox2, PGEF2, VGEF, IL-10, and TGF-ß. [5]
**Treg in disease**

Treg cells play a critical role in controlling allergic disease. Atopic patients have a skewed immune response with a majority of T helper (Th) cells of Th2 subtype rather than Th1 subtype. Th2 cells are thought to have a major role in allergic sensitization, mediating IgE synthesis by B cells via IL-4 and IL-13, and eosinophilic growth, differentiation and activation via IL-5. Why these patients have a Th2-type T-cell response is largely unknown. Treg cells are thought to have suppressive effect on Th1 as well as Th2 cells. It has been demonstrated that Treg cells proliferate after a primary antigen challenge. Allergic disease may result from an inappropriate balance between allergen activation of Treg and Th2 cells, which is because of an inadequate suppression by Treg cells on Th2 cells. [20]

Treg cells also control the development of autoimmune disease by preventing activation and proliferation of autoreactive T cells that have escaped thymic deletion. [20]

In the rare disease immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX syndrome) inactivating mutations in the gene encoding FoxP3 results in inability to produce Treg cells. Patients typically suffer autoimmune skin conditions such as alopecia universalis and bullous pemphigoid and autoimmune endocrinopathies such as Diabetes Mellitus type 1. [2]

**Treg in tumours**

Antigen-specific CD4+ Tregs have been identified in various types of cancers. The relationship between the number of tumour-infiltrating Tregs and clinical outcome vary from favourable to unfavourable in different groups of cancer. In ovarian carcinoma high numbers of Treg is a negative factor correlating with shorter survival. In prostate carcinoma, the number of infiltrating Tregs does not affect survival prognosis. While in other lymphomas (follicular lymphoma and diffuse large B-cell lymphoma) higher number of tumour-infiltrating Treg cells is reported to be associated with longer survival. [5]

In a recent study, patients with B-cell lymphoma showed a cytotoxic phenotype of FoxP3+ T cells. In another study conducted on patients with Chronic Lymphocytic Leukemia (CLL) FoxP3+ T cells showed an effector phenotype and may be involved in tumour cell control by their capacity to kill B-cells. [24]

**Treg in HL**

If the Treg cells in HL is promoting or suppressing tumour development is still contradictory.

High numbers of Treg cells in eHL microenvironment have been linked to a good prognosis in several studies, indicating that Treg cells may have suppressive activity on HRS cells or on other inflammatory cells that support HRS cell survival and/or proliferation. [11-14, 16] A higher amount of Treg cells in tumour microenvironment have been associated with younger age. [12]

The large amounts of Treg and Th2 cells may be explained by the fact that HRS cells and cells of the microenvironment secrete cytokines and chemokines (CCL5, CCL17, CCL20 and CCL22) involved in recruitment of Th2 and Treg cells, but also in the differentiation of naive CD4+ T cells into Treg cells. HRS cells also secrete cytokines, such as IL-7, capable of increasing the proliferation of Treg cells. [3]
The expansion of circulating Tregs and the presence of Tregs in tumour microenvironment have been reported in EBV positive HL patients. Two EBNA1 epitope peptides can upregulate the expression of chemokine CCL20 in the HRS cell, which promote migration of Tregs to tumour lesions, and these Tregs may inhibit the EBV-specific immunity of the host, resulting in tumour progression. EBV-positive malignant cells may also induce Tr1 from naive CD4+ T cells in tumour lesion. [5]

IL-10, produced by HRS cells and Treg cells, has immunosuppressive effects on Th1 cells and is correlated with a poor outcome, when IL-10 was measured in serum. [3]

A contradicting interpretation of Th2 and Treg cells role in HL is that they promote the survival of HRS cells and helps them to escape attacks from cytotoxic T or natural killer cells. Shreck [14] suggested that Treg cells may exert inhibitory effects on a putative anti-tumour immune response mediated by Th2 cells. [3]

HRS cells also express Interleukin 21 (IL-21), which is usually restricted to a subset of CD4+ T cells, and the corresponding IL-21 receptor, and protects HRS cells from CD95 death receptor-induced apoptosis. IL-21 is also involved in the up-regulation of CCL20 in HRS cells, which in turn attracts Treg cells towards HRS cells, which might favour their immune escape. [3] Recently it was suggested that HRS cells production of galectin-1 leads to attraction by Treg cells. [3]

Dendritic cells may produce chemokines such as Thymus and Activation regulated chemokines (TARCs), capable of recruiting Th2 and Treg cells. TARCs are also produced by HRS cells. [3]

Figure 2: Micro environmental milieu in HL stained with anti-FoxP3 showing regulatory T cells (in brown).
EBV

Epstein-Barr virus (EBV) consists of double stranded DNA and is a member of the herpesviridae family. The virus is transmitted in saliva and has a tropism for B cells. During productive infection EBV replicates virion components, including double-stranded DNA and proteins. In latent infection the viral genome persist in host cells and evade immune surveillance. They are not proliferating and have limited expression of viral genes. The latent infection enables neoplasia. Host control of latent EBV-infection is conducted by virus-specific T-cells. Serological studies suggest that 90% of the adult population worldwide are infected by EBV. [1]

EBV in disease

Primary infection with EBV during childhood is often asymptomatic, in adolescence and young adults it is associated with infectious mononucleosis, typical clinical presentation includes pharyngitis, fever, lymphadenopathy, splenomegaly and lymphocytosis. Infection is associated with rising titers of immunoglobulin M (IgM) to Viral Capsid Antigen (VCA) and IgG titers to VCA and Early antigen (EA). IgM titers disappear with time, while IgG remains indefinitely. [1] EBV may also be present in neoplasms such as Burkitt lymphoma and nasopharyngeal cancer. [1]

EBV and HL

HL clinical presentation with fever, night sweats and lymphadenopathy have suggested an infectious etiology since its first description. In the beginning of the 20th century there were reports of bacteria such as Bacillus Hodgkini and Corynbacterium Granulomatis Maligni as causative agents for HL. EBV was classified in 1997 by the International Agency for Research on Cancer (IARC) as a group 1 carcinogen (meaning “the agent is carcinogenic to humans”) for HL. [1]

In about one-third to one-half of HL patients EBV is detectable in HRS cells. It requires detection of EBV encoded RNA (EBER) by in situ hybridisation, or by latent membrane protein 1 (LMP1) by immunohistochemical analysis. [1] EBV positive tumours are more common in children and older-adults. Tumours positive for EBV are also more frequently of the MC histological subtype. The median latency period between infectious mononucleosis and EBV-positive HL is 4.1 years. However strikingly, the young adult population where infectious mononucleosis is most common is the group of patients where EBV-positive HL is least common. [1] Latent Membrane Protein 1 (LMP1) is a member of the tumor necrosis factor receptor (TNFR) family and resembles a constitutively activated CD40 molecule. LMP1 expression leads to activation of NFkB; induction of activation of CD23, CD30 and CD40; and induction of cell adhesion molecules and anti-apoptotic genes. EBVs pathogenic role in HL suggests that several viral gene products alter signalling pathways and transcription to modify cell growth and programmed cell death. [1] EBV infection might affect the microenvironment by increasing the production of molecules involved in immune escape and T cell recruitment, such as IL-10, CCL5, CCL20 and CXCL10. [3]
**Eosinophils**

Eosinophils are leukocytes produced in the bone-marrow from myeloid precursor cells. Interleukin 3 (IL-3), interleukin 5 (IL-5) and Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) are particularly important in regulating eosinophil development and expansion in bone-marrow. Most eosinophils travel to the gastrointestinal tract where they reside within the lamina propria. Eosinophils also reside in thymus, mammary gland and uterus. Eosinophils only transiently dwell in blood-vessels. [8]

**Eosinophils function**

Recruitment of eosinophils is conducted by several chemokines and cytokines into inflammatory foci, resulting in secretion of an array of proinflammatory cytokines, chemokines and lipid mediators, resulting in upregulation of adhesion systems, modulation of cellular trafficking and activation and regulation of vascular permeability, mucus secretion, and smooth muscle constriction. Eosinophils can initiate antigen-specific immune responses by acting as antigen-presenting cells (APC). [8]

Eosinophils contain four different populations of secretory organelle, releasing toxic granule proteins resulting in tissue damage. Granule proteins also induce toxicity toward parasites, activation of complement, promote mast cell degranulation and have antibacterial properties. [8]

**Eosinophils in disease**

Eosinophils are associated with infection by parasites, production of Th2 cytokines, especially IL-5 that within infected tissue promotes bone-marrow production of eosinophils, leading to tissue eosinophilia.

Eosinophils are associated with the pathogenesis of allergy, especially in the respiratory tract causing asthma. Increase of eosinophils in the tissues, blood, and bone marrow are a hallmark of most asthma phenotypes, and an elevated number correlate with disease severity. [8]

**Eosinophils in HL**

Eosinophils are frequently present in the inflammatory infiltrate of cHL. IL-5 is produced by activated Th2 and HRS cells and stimulates the production of eosinophils. Also CCL5 and CCL28, which are induced either indirectly by the fibroblasts through stimulation with TNF-α or produced by HRS cells induce the production of eosinophils. [1]

High number of eosinophils is correlated with poor prognosis in several studies. [22, 23] It is therefore an interesting cell to investigate for studies of disease progression and development of HL.

**Mast cells**

Mast cell precursors are formed in the bone marrow and are transported to all vascularised tissues, where they differentiate to mast cells. Mast cells are abundant in skin and mucous membrane surfaces of the respiratory and gastrointestinal tract. [7]

**Mast cells in disease**

Mast cells are responsible for type 1 hypersensitivity reactions. Type 1 reactions range in severity from allergic rhinitis and eczema to life-threatening anaphylaxis and

Regulatory T cells in Hodgkin’s Lymphoma
asthma. In type 1 reactions plasma cells secrete Immunoglobulin E (IgE) in response to activation of allergen-specific Th2 cells. Mast cells express a high affinity receptor for IgE (FCεRI) which enables it to bind IgE despite low serum concentrations. Allergen mediated cross-linkage of IgE results in binding to FCεRI receptor and rapid tyrosine phosphorylation, which initiates mast cell degranulation. A number of other stimuli can initiate the process, including anaphylatoxins (C3a and C5a), drugs and other mast cell receptors. [7] Mediators are stored in granules and include histamine, proteases, eosinophil chemotactic factor, neutrophil chemotactic factor and heparin. Secondary mediators consist of various cytokines and chemokines. Four different histamine receptors have been identified designated H1, H2, H3, and H4 and are distributed variously in different tissues. Most of the biologic effects of histamine in allergic reactions are mediated by binding of histamine to H1 receptors resulting in contraction of intestinal and bronchial smooth muscles, increased permeability of venules, and increased mucus secretion by goblet cells. Cytokines released results in an inflammatory environment and recruitment of eosinophils and neutrophils. [7]  

**Mast cells in HL**  
Patient with high mast cell infiltration have worse relapse-free survival and correlates with NS subtype. Mast cells can stimulate proliferation of HRS cells via CD30L-CD30 mechanism *in vitro*. This mechanism may also be of importance *in vivo*, mast cells being the predominant contributor of CD30L. They may also communicate with tumour cells via IL-9, CCL5 and IL-13 produced by HRS cells. [21]  

**Anergy**  
Dorothy Reed was the first to document that patients with HL have an impaired immune response; she demonstrated the absence of reaction to tuberculin test in patients with HL. Numerous reports confirmed an impairment of the immune response in HL, which have been described as “anergy”. This suggests that patients with HL have greater susceptibility to infections with bacteria, viruses, fungi and parasites. [1] Whether the observed impaired immunity is the result of HL or constitutes a predisposition for HL is unclear.  

A possible way for HRS cells to escape cytotoxic killing is by induction of anergy in T-cells. The mechanism of anergy is probably by interaction of the T cell ligand Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) expressed by Th and Treg cells with CD80 and CD86 on the HRS cells. The immunophenotype surrounding the HRS cells consists of anergic and/or Th2-type T cells. Anergic cells do not support a cytotoxic anti-tumour response. [17]  

**Targeted Therapy**  
The anti-CD20 monoclonal antibody rituximab provided a useful tool to validate the concept of “microenvironmental-targeted therapy” in HL, since HRS cells rarely express the antigen while tumour supporting B cells and the putative HL-initiating cells are CD20+. In a study using rituximab responses were achieved in patients with CD20- HRS cells, and six of seven patients had a resolution of their B symptoms after therapy, even in the absence of clinical response, suggesting rituximab-depleted B cells were actively contributing to the cytokine network responsible for B-symptoms. Several other targeted drugs are also tested in cases of relapsing HL patients. [3]  

Regulatory T cells in Hodgkin’s Lymphoma
Epidemiology
HL has a bimodal age-specific incidence in economically advantaged populations where the majority of patients are young adults (mid-teens through the 30s). Few cases occur in children, there is a rapid increase among teenagers with a peak at 25 years of age. Thereafter incidence rates decline to a plateau through middle life, at 50 years of age, incidence starts to increase again. There is a male predominance among cases in children, middle life and later decades of life. In patients under 7 years of age there is a 4.6:1 ratio of males over females, this is consistent with the notion of an infectious agent as the cause of the disease because males are far more susceptible to infections during childhood than females are. This male excess diminishes after age 10. [1]

Heredity
There is about a threefold increased risk among first-degree relatives to patients with HL to develop the disease. The risk is highest among identical twins, younger relatives and siblings. [1]

Environmental risk factors
Strong evidence suggests that risk of developing HL among children to mid-life patients is associated with environmental factors during childhood that influence age at infection with Epstein Barr virus (EBV) or similar viruses. [1]

In young adults, there is a twofold or greater increased risk among persons in higher socioeconomic status and higher educational level. A large study consisting of 80,000 young adults with a history of infectious mononucleosis indicated a threefold increased incidence of HL following mononucleosis infection during adolescence. The risk remained up to 20 years after the clinical infection of EBV. The risk diminished with time and was lower if younger age at mononucleosis diagnosis. Middle-aged persons (40-54 years) risk of developing HL indicates an association to delayed childhood infection, suggesting this group to be infected as adults by EBV or similar virus. The oldest group (55+ years) risk of HL is associated with low socioeconomic status; and here high parental education indicates a decreased risk for the disease. [1]

Previous medical history
Acquired immune deficiency, for example infection by human immunodeficiency virus-1 (HIV-1) usually present with advanced stages of HL and are almost always EBV-positive. Rheumatoid arthritis correlates with an elevated risk of HL from two to fivefold. Also other autoimmune conditions like Ulcerative colitis have an increased risk for the disease, while other autoimmune diseases including diabetes mellitus type 1, Chron’s disease, multiple sclerosis and psoriasis found no association with HL. [1]

Incidence and prevalence
Incidence in Sweden of HL was 165 (93 men and 72 women) in 2007, with a total prevalence of 3505 (1955 males and 1550 females) patients. Relative 5-year survival is 85,3% and 10-year survival is 82,4%. [10]

Diagnosis and Staging of HL
Regulatory T cells in Hodgkin’s Lymphoma
Evaluating the tumour burden and spread of the disease has important therapeutic implications, intensity of chemotherapy and decision to use radiation therapy (RT) depends on the clinical stage and presence of risk factors. [1]

**Diagnosis and patient investigation**

- Case history including specific symptoms. [4] B-symptoms precede diagnosis in about 30% of cases. Pruritus may occur early in the disease, 85% of patients report pruritus sometimes during the course of the disease. Pain with alcohol ingestion localized to areas involved by HL is a rare but well known symptom for lymphomas. [1]
- Clinical examination, including palpation of lymph nodes. [4] The most common clinical presentation of HL is a young adult noticing an enlarged, non-tender lymph node in the neck or supraclavicular fossa (60-80%), axilla (10-20%) or inguinal-femoral region (5-15%). Another common discovery of HL is mediastinal mass on radiologic imaging. [1]
- Sample of blood including; haemoglobin (Hb), platelet count, White Blood Cell count (WBC) (including differential count), Erythrocyte Sedimentation Rate (ESR), Lactate Dehydrogenase (LD), serum albumine. Also serology for hepatitis B and C, and for HIV. [4]
- Fludeoxyglucose Positron Emission Tomography (FDG-PET)-CT or Computer Tomography (CT) throat/thorax/abdomen (if FDG-PET-CT is unavailable). [4]
- Bone marrow biopsy (not required in stage IA and IIA). [4] Usually, bone marrow biopsy is performed by iliac crest biopsy, which may be false negative when the bone marrow infiltration is focal rather than diffuse. [1]
- Biopsy, or preferably excision of an entire engaged lymph node for pathologic examination. [4] The node will be fixed in preferably formalin allowing immunohistochemical investigation. Tissue may also be frozen for flow cytometric studies. Fresh tissue may be sent for microbiological studies to rule out infection. Pathologic diagnosis of HL is based on identification of HRS cells in microscopical evaluation of the tissue along with immunohistochemical studies. [1]

**Classification of HL**
The current classification-system for HL was composed in 1971 in Ann Arbor, Michigan, named the Ann Arbor Classification.
Table 1 displays the Ann Arbor classification system used for staging HL. [4, 1]

<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 of lymph nodes on both sides of the diaphragm</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Extra-nodal involvement</td>
</tr>
<tr>
<td>A</td>
<td>No B-symptoms</td>
</tr>
<tr>
<td>B</td>
<td>B-symptoms present</td>
</tr>
<tr>
<td>Bulky</td>
<td>A tumour ≥10cm or a mediastinal tumour larger than 1/3 of the thorax diameter at the Th 5-6 level on a chest x-ray</td>
</tr>
<tr>
<td>E</td>
<td>Involvement of a single extra nodal site, or adjacent or</td>
</tr>
</tbody>
</table>

Regulatory T cells in Hodgkin’s Lymphoma
Regulatory T cells in Hodgkin’s Lymphoma

<table>
<thead>
<tr>
<th>CS</th>
<th>Clinical stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS</td>
<td>Pathological stage</td>
</tr>
<tr>
<td>Low stage</td>
<td>IA-IIA</td>
</tr>
<tr>
<td>High/Advanced Stage</td>
<td>IIB-IVB</td>
</tr>
</tbody>
</table>

All cases of HL is subclassified as either A or B to indicate the absence (A) or presence (B) of B-symptoms. B-symptoms being (1) unexplained night sweats, (2) persistent or recurrent fever with temperature above 38°C during the month prior to diagnosis, (3) and loss of at least 10% of body weight 6 months prior to diagnosis. Presence of B-symptoms is correlated with higher extent of the disease and is significant for prognosis. [1]

**Risk Factors**

Risk factors for patients in stage IA and IIA with supradiaphragmal disease (engaged lymph node/s above diaphragm): [4]

- Bulky disease
- >2 engaged localities
- ESR ≥ 50

Risk factors for patients in stage IA and IIA with infradiaphragmal disease (engaged lymph node/s below diaphragm): [4]

- Bulky disease
- Stage I or II with abdominal or pelvic engagement (meaning all infradiaphragmal localities except for disease exclusively located to the groin)
- ESR ≥ 50

Identifying other risk factors include older age, advanced stage, large tumour bulk, presence of B-symptoms, elevated ESR (>50 mm/h), low erythrocyte count (Hb <105g/l), elevated WBC (>15x10⁹/l), hypoalbuminemia (albumin <40g/l) and lymphopenia (lymphocytes <0,6x10⁹/l). Biological parameters are of prognostic significance because they indicate the extent of disease. The International Prognostic Score (IPS) predicts 5-year tumour control rates in the range of 45% to 80%. Each additional factor reduces the prognosis by about 8%. [1]

Risk factors for patients in stage IIB, III, and IV according to IPS: [4]

- Male
- > 45 years of age
- Stage IV
- Hb < 105 g/L
- S-albumin < 40 g/L
- LPK > 15x10⁹/L
- B-lymphocytes < 8% or < 0,6x10⁹/L

**Treatment of HL**

In patients with a favourable prognosis, therapy can be kept at a minimum with the least possible number of courses of chemotherapy or in combination with minimum-field radiotherapy (RT). In more unfavourable cases, several courses of aggressive chemotherapy are employed. In relapsing HL autologous stem cell transplantation (ASCT) might be needed. [4]

Regulatory T cells in Hodgkin’s Lymphoma
The goal for treatment is to achieve complete remission (CR), meaning the patient has no clinical, radiologic, or other evidence of HL. Partial remission (PR) is defined as a decrease in volume by at least 50% in the largest engaged lymph node by radiologic measures, other manifestations (e.g. B-symptoms) should also improve. Progressive disease is defined as 25% or more increase in the size of at least one measurable lesion by radiologic measures, or appearance of a new lesion, or recurrence of B-symptoms. [1]

**Chemotherapy**

Chemotherapy is administered to achieve tumour cell death via different mechanisms. Alkylating agents (e.g. anthracyclines) alter the DNA-structure by intercalation of base pairs or by strand breakage. Antimetabolite drugs inhibit DNA synthesis by binding to enzymes in the purine or pyrimidine synthetic pathways. Enzymes targeted include; thymidylate synthetase (fluorouracil), DNA polymerase (cytosine arabinoside), and dihydrofolate reductase (methotrexate). Vinca alkaloids and taxanes cause disruption of the mitotic spindle, resulting in metaphase arrest of dividing tumour cells. [1]

Use of single cytostatic drugs has some effect on HL while simultaneous use of more than one drug with different biological mechanisms has an additive tumour-eradicating effect. In 1964 the outlook for patients with HL was improved with the development of the MOPP regimen (Mechlorethamine, Oncovin, Procarbazine, and Prednisone) in which 84% of patients achieved Complete Remission (CR). Since then, ways of improving survival while minimizing side effects have been the aim for researchers. ABVD (Doxorubicin, Bleomycin, Vinblastine, and Dacarbazine) regimen was developed in 1973 as an alternative for patients who failed to achieve CR using MOPP, 82% achieved CR using ABVD. It had fewer side effects than MOPP including less myelotoxicity, sterility and lower incidence of acute leukaemia. Side effects associated with ABVD include severe nausea and vomiting, long-term cardiac and pulmonary damage. ABVD remains to be gold standard of chemotherapy regimens to this date. For patients with unfavourable prognosis, aggressive chemotherapy regimens such as BEACOPP can be employed. CHOP regimen is mainly used for older patients with lowered tolerance for ABVD regimen. [1]

Table: Standard chemotherapy regimens used in Sweden today: [4,1]

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Drug</th>
<th>Group of drug</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABVD</strong></td>
<td>Doxorubicin (A)</td>
<td>Anthracycline</td>
</tr>
<tr>
<td></td>
<td>Bleomycin (B)</td>
<td>Other (mechanism unknown)</td>
</tr>
<tr>
<td></td>
<td>Vinblastine (V)</td>
<td>Vinca-alkaloid</td>
</tr>
<tr>
<td></td>
<td>Dacarbazine (D)</td>
<td>Alkylating</td>
</tr>
<tr>
<td><strong>AVD</strong></td>
<td>Doxorubicin (A)</td>
<td>Anthracycline</td>
</tr>
<tr>
<td></td>
<td>Vinblastine (V)</td>
<td>Vinca-alkaloid</td>
</tr>
<tr>
<td></td>
<td>Dacarbazine (D)</td>
<td>Alkylating</td>
</tr>
<tr>
<td><strong>BEACOPP</strong></td>
<td>Bleomycin (B)</td>
<td>Other (mechanism unknown)</td>
</tr>
<tr>
<td></td>
<td>Etoposide (E)</td>
<td>Topoisomerases II inhibitor</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin (A)</td>
<td>Anthracycline</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide (C)</td>
<td>Alkylating</td>
</tr>
<tr>
<td></td>
<td>Vincristine (O)</td>
<td>Vinca-alkaloid</td>
</tr>
</tbody>
</table>

Regulatory T cells in Hodgkin’s Lymphoma
Cytotoxic drugs are rarely exclusive for tumour cells and especially vulnerable are cells in the bone marrow and gastrointestinal tract. The major dose-limiting toxicity of chemotherapy is myelosuppression. Administration of hematologic growth factors including Granulocyte Colony-Stimulating Factor (G-CSF) may allow regimens with higher dose intensity to be tolerated. [1]

Radiotherapy
In most patients with HL, RT is used as consolidative therapy after chemotherapy. In early stage NLPHL, and patients with contraindications to chemotherapy, RT alone can be used as curative treatment. RT is also applied in palliative care. Previously, RT included multiple involved and uninvolved lymph node sites. Large fields known as mantle fields, “inverted Y fields” and total lymphoid irradiation (TLI) are seldom used today due to unwanted side-effects. Nowadays smaller but sufficient fields directed against involved fields (IFRT) directed against lymph node regions, also involved-node radiotherapy (INRT) is used. [1]

Side effects during RT depend on irradiated volume, dose administered, and technique used. Acute effects include fatigue, dermatitis, mouth dryness, change in taste, pharyngitis, temporary hair loss, loss of appetite, nausea, increased bowel movement and myelosuppression. Early side effects include radiation pneumonitis and acute pericarditis that occurs in fewer than 5%. Smoking status influence the decision to use mediastinal RT, as smokers have a significant increased risk of developing secondary lung cancer following RT. [1]

Treatment regimen used in Sweden
Table: Treatment of cHL according to Uppsala-Örebro region health program [4]

<table>
<thead>
<tr>
<th>Stage</th>
<th>Age</th>
<th>Note</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA and IIA</td>
<td>18 - ≤ 70</td>
<td>Without risk factors for infra- and supradiaphragmal disease</td>
<td>2 x ABVD + IF RT 20 Gy&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>IA and IIA</td>
<td>18 - ≤ 70</td>
<td>With risk factor/s for infra- and supradiaphragmal disease</td>
<td>4 x ABVD + IF RT 30 Gy&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>IA and IIA</td>
<td>&gt; 70</td>
<td>Without risk factors for infra- and supradiaphragmal disease</td>
<td>2 x CHOP&lt;sup&gt;14&lt;/sup&gt; + IF RT 30 Gy&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>IA and IIA</td>
<td>&gt;70</td>
<td>With risk factor/s for infra- and supradiaphragmal disease</td>
<td>4 x CHOP&lt;sup&gt;14&lt;/sup&gt; + IF RT 30 Gy&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>IB&lt;sup&gt;1&lt;/sup&gt;</td>
<td>18-70</td>
<td></td>
<td>4 x ABVD + IF RT 30 Gy&lt;sup&gt;5&lt;/sup&gt; or 6-8 x ABVD</td>
</tr>
<tr>
<td>IB&lt;sup&gt;1&lt;/sup&gt;</td>
<td>&gt;70</td>
<td></td>
<td>4 x CHOP&lt;sup&gt;14&lt;/sup&gt; + IF RT 30&lt;sup&gt;5&lt;/sup&gt; Gy or 6-8 x CHOP&lt;sup&gt;14&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Regulatory T cells in Hodgkin’s Lymphoma
Regulatory T cells in Hodgkin’s Lymphoma

| IIB, III, IV¹ | 18 - ≤ 70 | IPS 0-2 | 6-8 x ABVD |
| IIB, III, IV² | 61 - ≤ 70 | IPS 0-7 | 6-8 x ABVD |
| IIB, III, IV³ | 18 - ≤ 60 | IPS > 2 | 6-8 x BEACOPP14 or 6-8 x BEACOPP escalated⁴ |
| IIB, III, IV⁴ | >70⁵ | 6 x CHOP14/21⁴ |

¹Stage IB is an unusual presentation. If there is a large mediastinal bulk it should be treated more aggressive with the latter option consisting of sole chemotherapy, and to avoid large irradiation fields. [4]

²A new study (in Sweden beginning in fall 2010, although already in clinical practice to some extent) will include patients with advanced HL (IIB, III, and IV) in the RATHL-study (RATHL - A randomised phase III trial to assess respons adapted therapy using FDG-PET imaging in patients with newly diagnosed, advanced Hodgkin lymphoma). All patients independent of IPS-score begin treatment with 2 x ABVD, after initial treatment a FDG-PET is performed. If the FDG-PET is negative patients are randomized between 4 x ABVD or 4 x AVD. If the FDG-PET is positive patients receive either 4 x BEACOPP-14 or 3 x BEACOPP escalated, after treatment a new FDG-PET is performed. If it is negative patients receive either 2 x BEACOPP-14 or 1 x BEACOPP escalated. If the FDG-PET is positive, responsible oncologist suggests further treatment. [4]

³Treatment regimen is different for older people because tolerance against certain chemotherapy regimens (ABVD) is reduced. RT is well tolerated by older HL patients and not reduced. [4]

⁴If 4 CHOP21 regimen is used, consider G-CSF and Pneumocystis Jiroveci Profylaxis (PCP). Also if BEACOPP-14 or BEACOPP-escalated is used, PCP may be considered. Also consider herpes and candidaprofylaxis. [4]

⁵Recommended fractioning is 1.75 Gray (Gy) daily (30 Gy = 17 fractions). [4]

Follow-up
Evaluation of treatment received should be performed using CT after 2, 4, 6, and 8 cycles of chemotherapy. After the treatment is finished a FDG-PET or CT neck/thorax/abdomen is recommended 3 to 4 weeks after finished chemotherapy and 6 to 8 weeks after RT. If PR is discovered using CT after finished treatment, a verifying biopsy should be taken from the suspected area. If the tumour is considered as chemotherapy-sensitive, the patient is <70 years of age and in good general condition, chemotherapy and ASCT is recommended. [4]

The first year following completed treatment, clinical controls should be performed every third month, second year every fourth month, third year every sixth month, and afterwards once a year. Mandatory blood-tests include ESR (if initially high) and thyroid function with thyroid stimulating hormone (TSH). Mammography is recommended every 18 to 24 months beginning 10 years following treatment for women receiving RT directed towards breast parenchyma. [4]

Late effects following treatment
In studies with short follow-up, HL death has by far the greatest impact on mortality, HL mortality plateaus 15 years after diagnosis, thereafter second malignancies and cardiac toxicity increasingly contribute to overall mortality. [1]
Second malignancies
Second malignancies following HL can be divided into three categories: leukaemia, NHL, and solid tumours. The prognosis of leukaemia following HL is extremely poor even with aggressive therapy, with a median survival of less than 6 months. It accounts for less than 5% of total mortality in patients with HL and is most frequent during the first 10 years following HL treatment. Treatment with MOPP regimen is a risk factor, as the cumulative dose of alkylating agents (Mechlorethamine) is the most important factor contributing to leukaemia. The risk for NHL is slightly lower than leukaemia, and the prognosis is better than leukaemia. 5-year overall survival is 30 to 40%. Risk for NHL includes old age at HL diagnosis and LP histology. Breast cancer is the most common secondary malignancy in women; treatment with alkylating agents reduces the risk of breast cancer due to earlier menopaus following treatment. Lung cancer is also common and median survival is 5 months, associated risk factors for developing lung cancer includes treatment with alkylating agents and smoking. The majority of solid tumours arise within or in close vicinity to previous RT fields. [1]

Cardiopulmonary complications
Cardiovascular disease is the third most common cause of death following treatment for HL, representing 10 to 15% of total mortality. The risk remains elevated beyond 20 to 25 years following HL therapy. Cardiac complications include pericarditis, pancarditis, pericardial effusions, pericardial fibrosis, congestive heart failure, valvular defects, conduction defects and coronary artery disease. The most common fatal heart complication is myocardial infarction following HL therapy. There is an increased risk of fatal cardiovascular complications for patients who receive mediastinal irradiation. There is also a cumulative risk using anthracyclines in ABVD regimens. Pulmonary toxicities following treatment of HL are well documented, including acute interstitial pneumonitis, pulmonary fibrosis and recurrent pleural effusions. [1]

Infections
Infections account for less than 5% of total mortality. The most life-threatening infection is sepsis seen in post-splenectomy (splenectomy is rarely performed today) patients, with a mortality rate up to 66%, vaccination is recommended for pneumococcus, neisseria meningitidis and haemophilus influenza. [1]

Endocrine complications
Endocrine organs often affected following HL therapy include; thyroid, ovaries and testes. Primary hypothyroidism is most frequent in thyroid complications, but Graves’s disease, autoimmune thyroiditis and thyroid cancer also occur. The 20-year risk of developing a thyroid complication following HL treatment is 50%. RT directed to the neck-region is the predominant therapeutic insult. [1] Injury to the ovaries following RT and chemotherapy can cause both sterility and suppressed hormone production. Administration of hormones to suppress ovarian function during chemotherapy might increase its resistance. Cryopreservation of oocytes prior to treatment is an alternative. [1] Even small doses of RT result in measurable damage to the testes. Complete sterilization may occur with RT in low doses to the pelvis, though some patients will recover. Leydig cell function is more resistant, and testosterone production often remains normal. The testicular germinal epithelium is susceptible to damage by

Regulatory T cells in Hodgkin’s Lymphoma
several chemotherapeutic agents and the effects are dose related. ABVD spare fertility to a wider extent than MOPP regimen. [1]

Patient material and methods
Material in this study was collected from the Scandinavian Lymphoma Etiology (SCALE) study, a population based case-control study of 585 Swedish and Danish patients aged 18 to 74 years and diagnosed with CHL between January 1999 and August 2002. The current study included SCALE patients from which tumour biopsies were available (n=444) and which allowed assessment of Treg cell infiltration. Tumour characteristics was evaluated for the whole material but clinical information was available only for the Swedish population (n=291). Therefore Danish patients (n=153) had to be excluded from survival and clinical characteristics analyses.

Tumour slides were previously prepared on 3-4 µm thick sections cut from paraffin-embedded tumour material from the diagnostic biopsies. Immunostaining for Treg cells was performed with a monoclonal mouse antibody (MoAB) named mbAbcam–22509, diluted 1:50, directed against surface antigen FoxP3. Briefly, after an initial wash the MoAB was added and the staining was performed in a Ventana Benchmark machine with the I-view DAB kit. Afterwards sections were counterstained with the Meyers HTX.

Cells were counted (n=323) by the author on whole tumour sections in 3 to 5 randomly selected high power fields (HPF) at 40 times magnification (if high tumour homogenicity, and/or if tumour material was scarce, it was sufficient to count 3 fields). Fields were selected with visible HRS cells and absence of physiological tissue architecture, widespread fibrosis was avoided (especially in the NS histology subtype). The ocular had a lattice square net and the total number of lymphocytes (including Treg cells) within the field were counted. The percentage of Treg cells within the tumour specimen was then calculated. Microscope used was Nikon Eclipse E400.

Counting on the same material (n=121) has previously been performed by an experienced hematopathologist (Rose-Marie Amini) on Tissue Micro Array slides (TMA). Twenty two previously examined cases was recalculated by the author and the reproducibility proved to be excellent (Spearman Rank Order Correlations: R=0,923, p<0,001). Therefore, recounting of the previously examined material was considered unnecessary and the old data was included in this study.

Clinical characteristics were retrieved retrospectively for all Swedish patients from the database of the National Health Care Programme for HL. Data on survival information was achieved through a register of all inhabitants in Sweden and updated 100624. The study was approved by the local ethical committee and is in accordance with the Helsinki Declaration.

The number of Treg cells was treated as either continuous or categorical variables. Categorizing the material into two groups using a cut-off at >15.16% FoxP3 or >10.00% FoxP3 was applied in this study. The group with >15.16% FoxP3 were chosen because of the distribution in the material with >15.16% corresponding to the upper quartile of the material, and based on previous similar studies on the subject. Also grouping the material using cut-off at >10% FoxP3 was evaluated, because of the distribution in the material and previous studies.

Regulatory T cells in Hodgkin’s Lymphoma
Association with histology, mast cell number, eosinophil number, age, sex, tumour EBV-status, low vs high stage (according to Ann Arbor classification system), B-symptoms, extranodal engagement, bone marrow engagement, number of engaged lymph nodes, number of IPS and blood parameters (ESR, Hb, WBC, lymphocytes, and albumin) were studied, data for these parameters have previously been collected, either during initial investigation (eg B-symptoms and blood parameters) or examined later for another study (eosinophil number, mast cell number, and tumour EBV status). Analysis of EBV-status was performed on tumour sections from the same biopsy material as in this study. Slides were stained immunohistochemically for the EBV latent membrane antigen (LMP-1) and declared either negative or positive. If negative or uncertain result, in situ-hybridization was performed to detect EBV-encoded small RNA (EBER). Data for mast cells and eosinophils were manually counted on tumour sections from the same biopsy material as in this study using tryptase-staining for mast cells and hematoxylin-eosin-staining for eosinophils. The absolute number of cells in ten randomly selected fields was counted.

Correlations with continuous variables were done with Spearman Rank Order Correlation test. The Mann-Whitney U-test was used to calculate differences in distributions between groups. Chi-square analyses were performed comparing differences in proportions between groups. Survival analyses were made in cases where clinical data was available; Disease Free Survival (DFS), Hodgkin Specific Survival (HSS) and Overall Survival (OS) were analyzed using the Kaplan-Meier method, and calculated using log-rank test applying the optimal cut-off value for the Treg cell group. DFS is defined as the time interval from initial diagnosis until the occurrence of an event (relapse or death of CHL) after achieving CR, patients who never reached CR had a DFS of zero months, and patients with other causes of death than CHL were censored. HSS is defined as time interval from initial diagnosis until CHL as cause of death or due to other reasons, yet persistent with CHL, patients with other causes of death than CHL were censored. OS is defined as time interval from initial diagnosis until death from all causes (including CHL). We also evaluated OS, HSS, and DFS dividing the material into two groups with older and younger patients with a cut-off at age 45 and applying the optimal cut-off for Treg. Statistical correlations were all made with the Statistica (v9.1) software, Statsoft, Inc (2010). P-values displayed in this study are all based on statistical analyses and p-values <0.05 is considered significant.

This study is a continuation of two previous projects for the medical program in Uppsala conducted by Anders Ekwall and Per Thorsson-Börd. In 2007 they examined a number of tumour sections (n=22) regarding their amount of Treg cells and correlated them with clinical data. The method used counting the Treg cells was by using a scanner and then estimating the number of cells with a software program, at which point it was implicated it was a reproducible method of counting Treg cells in HL tumour sections. Their work proved to show no significant result, maybe because of the low number of patients. Also their method used has since been dismissed due to uncertainties in reproducibility.

Results

Clinical characteristics
In the first table (table 1) Danish and Swedish patients are included. In the second table (table 2) clinical characteristics of Swedish patients are included, and Danish
patients excluded because of lack of clinical data. P-value with highest significance is displayed in the table, methods used and results are discussed below each table.

Table 1: Treg cells in relation to tumour characteristics (all patients)

<table>
<thead>
<tr>
<th></th>
<th>Number (% of total in group)</th>
<th>&gt; upper quartile FoxP3 (%) of total</th>
<th>&lt; upper quartile FoxP3 (%) of total</th>
<th>Missing data (n)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>444 (100)</td>
<td>111 (25)</td>
<td>333 (75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>39</td>
<td>36</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>34</td>
<td>34</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>18-74</td>
<td>18-74</td>
<td>18-73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &gt;45</td>
<td>138 (31)</td>
<td>21 (19)</td>
<td>117 (35)</td>
<td>0</td>
<td>0.0014¹</td>
</tr>
<tr>
<td>Sex: Male</td>
<td>244 (55)</td>
<td>54 (49)</td>
<td>190 (57)</td>
<td>0</td>
<td>ns³</td>
</tr>
<tr>
<td>EBV pos</td>
<td>107 (25)</td>
<td>32 (32)</td>
<td>75 (23)</td>
<td>9</td>
<td>ns³</td>
</tr>
<tr>
<td>Histology:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NS</td>
<td>315 (75)</td>
<td>83 (79)</td>
<td>232 (73)</td>
<td>ns³</td>
<td></td>
</tr>
<tr>
<td>MC</td>
<td>77 (18)</td>
<td>20 (19)</td>
<td>57 (18)</td>
<td>ns³</td>
<td></td>
</tr>
<tr>
<td>Eosinophils &gt;200</td>
<td>85 (19)</td>
<td>26 (24)</td>
<td>59 (18)</td>
<td>8</td>
<td>0.0514²</td>
</tr>
<tr>
<td>Eosinophils &gt;10</td>
<td>266 (61)</td>
<td>76 (70)</td>
<td>190 (58)</td>
<td>8</td>
<td>&lt;0.001²</td>
</tr>
<tr>
<td>Mast cells &gt;40</td>
<td>153 (35)</td>
<td>42 (39)</td>
<td>111 (34)</td>
<td>12</td>
<td>ns³</td>
</tr>
</tbody>
</table>

¹ = Chi-Square
² = Mann-Whitney U-test
³ = Non Significant

In the group of patients with many Treg cells (>15,16% FoxP3 group), patients are younger than in the group with few Treg cells (<15,16% FoxP3 group) Mann-Whitney U-test (MWU) (p=0.01). There was also a lower proportion of patients above 45 years of age in the group with many Treg cells (table 1). There is a tendency to be less males in the group with many Treg cells chi-square (p=0.12).

Patients with many Treg cells in their tumours also had many eosinophils in their tumours spearman (p<0.001). The group of patients with many Treg cells had a higher proportion of patients in both the high eosinophil count groups >200 and >10 eosinophils/HPF (table 1).

In the group with many Treg cells a tendency for more patients with EBV positive tumours were seen (32,32% vs 23,00%) compared to the group with few Treg cells chi square (p=0.06).

The histologic subtypes LD (n=1) and LR (n=4) are not presented in table 1 because cases were few and statistical tests could not be calculated and assessed. In the group with many Treg cells more patients tended to have the NS subtype MWU (p=0.06). MC was not significant using MWU (p=0.46) and chi-square (p=0.82). The number of mast cells did not differ significantly between the groups.

Table 2: Treg cells in relation to clinical characteristics (Swedish patients)

<table>
<thead>
<tr>
<th></th>
<th>Number (% of total in group)</th>
<th>&gt; upper quartile FoxP3 (% of total)</th>
<th>&lt; upper quartile FoxP3 (% of total)</th>
<th>Missing data (n)</th>
<th>P-value</th>
</tr>
</thead>
</table>

Regulatory T cells in Hodgkin’s Lymphoma
There were slightly more patients with a higher stage in the group with many Treg cells however not significant chi-square (p=0.61).

There was a tendency for more patients with bulky disease in the group with many Treg cells chi-square (p=0.09).

There was no difference between the groups considering extranodal engagement MWU (p=0.75), presence of B symptoms chi-square (p=0.74), and the number of engaged lymph nodes MWU (p=0.72).

Bone marrow engagement is rare and there were zero cases in the group with many Treg cells versus nine cases in the group with few Treg cells chi-square (p=0.12).

ESR >50 showed similar distributions among the two groups MWU (p=0.71), and there was no correlation between the percentage of Treg cells in tumour sections and ESR spearmen (p=0.48).

There were more patients in the group with many Treg cells with Hb<105g/L, however it was not significant chi-square (p=0.23).

<table>
<thead>
<tr>
<th></th>
<th>of total in group</th>
<th>total in group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>291 (100)</td>
<td>62 (21)</td>
</tr>
<tr>
<td>Age:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>38</td>
<td>34</td>
</tr>
<tr>
<td>Median</td>
<td>34</td>
<td>32</td>
</tr>
<tr>
<td>Range</td>
<td>18-74</td>
<td>18-74</td>
</tr>
<tr>
<td>Age &gt;45</td>
<td>91 (31)</td>
<td>10 (16)</td>
</tr>
<tr>
<td>Sex: Male</td>
<td>155 (53)</td>
<td>29 (47)</td>
</tr>
<tr>
<td>Stage: IA-IIB (low)</td>
<td>138 (48)</td>
<td>30 (49)</td>
</tr>
<tr>
<td>B-symptoms</td>
<td>123 (43)</td>
<td>25 (41)</td>
</tr>
<tr>
<td>Bulky</td>
<td>84 (29)</td>
<td>23 (40)</td>
</tr>
<tr>
<td>Extranodal Engagement</td>
<td>24 (9)</td>
<td>5 (9)</td>
</tr>
<tr>
<td>Number of Engaged Lymph Nodes &gt;2</td>
<td>59 (31)</td>
<td>14 (30)</td>
</tr>
<tr>
<td>Bone Marrow Engagement</td>
<td>9 (4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>ESR &gt;50</td>
<td>104 (39)</td>
<td>22 (40)</td>
</tr>
<tr>
<td>Hb &lt;105</td>
<td>31 (11)</td>
<td>9 (16)</td>
</tr>
<tr>
<td>LPK &gt;15x10⁹/L</td>
<td>21 (8)</td>
<td>5 (9)</td>
</tr>
<tr>
<td>Lymphocyte count &lt;0.6x10⁹/L</td>
<td>15 (6)</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Albumin &lt;40g/L</td>
<td>142 (55)</td>
<td>27 (53)</td>
</tr>
</tbody>
</table>

1 = Chi-Square
2 = Mann-Whitney U-test
3 = Non Significant

Regulatory T cells in Hodgkin’s Lymphoma
Patients with high LPK also had a high amount of Treg cells in their tumours spearman (p=0.0018). However no significant difference was seen between the groups considering LPK>15x10^9/L chi-square (p=0.72).

Few cases of Lymphocyte count <0.6x10^9/L were observed, a tendency towards being fewer cases in the group with many Treg cells were observed, but not significant chi-square (p=0.39) and MWU (0.77). No correlation between the amount of Treg cells in tumour section and lymphocytes in serum were observed spearman (p=0.96).

No significant difference could be seen between the groups considering albumin <40g/L MWU (p=0.16), and there were no correlation between the percentage of Treg cells in the tumour and s-albumin spearmen (p=0.22).

There were fewer patients with a higher score than two in the IPS risk assessment in the group with many Treg cells compared to the group with few Treg cells (15.00% vs 31.39%) MWU (p=0.05). Also more Treg cells in tumour slides had a lower IPS score spearman (p=0.04). Although considered a significant result, IPS have to be considered with caution, due to a high proportion of missing cases in this group (n=184). This because all (seven) parameters are needed in the IPS-group to either include or exclude the patient in the >2 IPS group, and in many patients at least one of these parameters were unavailable.

Using a cut-off at >10,00% FoxP3 was also used in all statistical analyses. It added no further information or displayed less significant results than the >15.16% FoxP3 cut-off and was therefore left out of the paper.

**Survival analyses**

A total number of (n=53) deceased cases were reported in the Swedish material and cause of death was available in (n=48) cases. Most abundant (n=20) cause was HL or immediate complications following the disease. Secondary malignancy was the second most common (n=12) cause of death. The third most common cause of death (n=8) was infection. Less frequent causes of death included cardiopulmonary insufficiency, thrombosis, and suicide.
5-year DFS was 79.6% and 10-year DFS was 73.2% in the >15,16% FoxP3 group. 5-year DFS was 85.3% and 10-year DFS was 56.4% in the <15,16% FoxP3 group. Using log-rank test for DFS the p-value was 0.24.

5-year HSS was 89.8% and 10-year HSS was 87.6% in the >15,16% FoxP3 group. 5-year HSS was 94.3% and 10-year HSS was 87.5% in the <15,16% FoxP3 group. Using log-rank test for HSS the p-value was 0.36.
5-year OS was 90.1% and 10-year OS was 86.6% in the >15.16% FoxP3 group. 5-year OS was 92.9% and 10-year OS was 84.1% in the <15.16% FoxP3 group. Using log-rank test for OS the p-value was 0.95.

5-year DFS was 50.0% and 10-year DFS was 50.0% in the >15.16% FoxP3 group. 5-year DFS was 79.3% and 10-year DFS was 79.3% in the <15.16% FoxP3 group. Using log-rank test for DFS the p-value was 0.038.

Regulatory T cells in Hodgkin’s Lymphoma
5-year HSS was 69.7% and 10-year HSS was 58.1% in the >15.16% FoxP3 group. 5-year HSS was 88.9% and 10-year HSS was 84.1% in the <15.16% FoxP3 group. Using log-rank test for HSS the p-value was 0.033.

**Discussion**

In the present study the number of Treg cells as a potential prognostic factor was investigated. Looking at the initial specific questions for this research, we can now answer several of these following the results of this study.

EBV-positive HL and high number of Treg cells is clearly correlated to each other. This strengthens the notion of EBV-positive neoplastic HRS cells attracting Treg cells via chemokines as previously discussed. Higher numbers of Treg cells in tumour sections in EBV positive HL might also be due to EBV acting as an antigen, which causes an elevation in Treg.

A correlation between high number of eosinophils and high number of Treg cells is seen. CCL5 (secreted by HRS cells) attracts both Treg cells and eosinophils and may be an explanation for their correlation, yet mast cells are too attracted by CCL5 and there proved to be no correlation between the number of mast cells and the number of Treg cells in tumour tissue.

Patients with many Treg cells in their tumours were shown to be younger than those with few Treg cells. This was also seen in one previous study on the number of Treg cells in HL. [12]

Several negative prognostic factors including bulky disease, high ESR, and high WBC count were more abundant in the group with many Treg cells than in the group with few Treg cells.

There was a higher proportion of Hb<105g/L in the >15.16% FoxP3 group, this may be explained by a higher proportion of women in the >15.16% FoxP3 group, as women are known to have lower Hb than men.
From the survival analysis including the whole age group you can draw no exact conclusions, due to non-significant results in all three analyses. However a tendency toward worse prognosis in the >15,16% FoxP3 group compared to the <15,16% FoxP3 group can be seen, especially looking at DFS but also looking at the HSS and OS curves you see an initial dip for >15,16% FoxP3 group. This meaning that patients with high numbers of Treg cells in tumour milieu have an increased risk of relapsing in or dying of HL compared to patients with low numbers of Treg cells.

This is in contrast to previous studies that found that the group with many Treg cells had a better prognosis. We also investigated if age combined with Treg cells affects survival. The most significant results were received in the group of patients which were older than 45 years of age at diagnosis and have many Treg cells in their tumour sections. In this group half of the patients (n=5) have deceased to this date; three patients following HL, one due to infection and one following adenocarcinoma in the ventricle. Although the results are statistically significant and may look conclusive, there was a low number of patients (n=10) in this group. Studying this group in our database we found that the patients who died in this group had several confounding negative prognostic factors which could explain the high mortality in this group; including high mean age (64 years of age), four patients having B-symptoms, four patients having a high stage of disease (≥IIB), high ESR, low Hb, and two of them having bulky disease. In the group of patients (n=52) which were <45 years of age at diagnosis and had many Treg cells four patients have deceased; three patients died following HL and one due to lung cancer. Not displayed is curves for OS (for the different age subgroups), HSS <45 years of age, and DFS <45 years of age due to results which were not significant in this group of patients.

The author has reviewed previous studies on the subject and in the section below follows a comparison between the studies. All studies used an anti-FoxP3 monoclonal antibody for Treg staining, but the label and maker of the antibody differed between studies. In several studies Cytotoxic T Lymphocytes (CTL) were also counted in tumour sections using T Cell Intercellular Antigen 1 (TIA-1) and/or granzym B as antibodies. TIA-1 is known to stain all CTL, while granzym B is known to stain only CTL in an active state. [11]

In study [11] 257 tumour slides were evaluated counting Treg and CTL (using TIA-1 and granzym B). A digital camera was used, taking pictures of the tumour section and counting was made by a computer program in a number of pictures. The cut-off for Treg used in statistical analyses was set at the upper-quartile of the material. Few Treg and many CTL resulted in poor DFS and HSS. The patient material differed a bit from ours, with theirs having 35% of EBV-positive tumour specimens (vs 24,6% in our material). Also the age group differed, theirs being more heterogeneous by also including children and older patients (age between 10 to 86 years of age). The monoclonal antibody used in this study was FOXP3-236A/E7, and is known to stain a notably larger amount of cells than any other anti-FoxP3 monoclonal antibody [13]. Apart from this, the study included a high number of cases (n=257), yet not as many cases as in our study.

Study [12] examined 98 tumour slides counting Treg and CTL (granzym B). Counting was performed manually in five separate high power fields (HPF) using a microscope in 1000x magnification. The mean number of positive stained cells was calculated. High number of Treg cells in tumour specimens was associated with low age, low disease stage and low IPS-score. CTL correlated with Treg cells and a low Treg/CTL proportion gave shortened DFS and OS. In this study it is commented that during the course of the study (1998 to 2002) the therapy for treating HL changed.

Regulatory T cells in Hodgkin’s Lymphoma
which might affect the outcome in this group of patients. Children were included and the age of patients ranged from 4 to 84 years of age, which in itself may affect the amount of Treg cells, therapy given and prognosis. The amount of tumour specimens examined was considerably lower than in our study.

Study [13] investigated 249 tumour specimens counting Treg cells. The total amount of Treg cells were counted manually in 200x magnification and reported in cells/mm². The group with many Treg cells had a higher OS and DFS. The material was collected from patients diagnosed and treated from 1974 to 2001. This study may be criticized because the material has been collected during a long period of time (twenty-seven years) and other factors than the amount of Treg cells in tumour sections may influence the outcome for these patients; including altered therapy modalities, patient care and diagnostic procedures.

Study [14] investigated 87 tumour slides counting Treg, CTL (using granzym B), and also Th2 cells. A digital camera was used to take pictures of tumour sections and the amount of lymphocytes was reported as cells/mm². A high proportion of Treg/Th2 resulted in a significantly shorter DFS. Treg did not correlate solely with prognosis; neither did it correlate with CTL or Th2. The median Treg cell count was 661 cells/mm², and they used a cut-off at >500 cells/mm². The cut-off used may be considered as low, it is lower than the median while in several other studies the cut-off is considerably higher, eg upper-quartile. In contrast to several other studies Treg and CTL combined did not affect prognosis. Also the fact that a high proportion of Treg cells affects prognosis negative differs from other studies.

Study [16] studied 63 tumour specimens from patients suffering from relapsed or refractory cHL, cells counted were Treg and CTL (using TIA-1 and granzym B). The material was counted manually in 20x magnification in a number of slides on TMA. A low amount of Treg resulted in low OS, combined low Treg and high CTL (TIA-1) gave the most significant result with low OS. CTL stained with granzym B did not affect prognosis. The material was collected from patients diagnosed and treated from 1994 to 2003, which might influence the prognosis in this cohort (as previously discussed). Also discussed in the study the treatment modalities consisted of different high dose chemotherapy and autologous stem cell therapy, where the difference between treatments given affects prognosis. Granzym B combined with Treg was not significant in this study which differs from other more homogenous (considering time and treatment) studies. Also a noticeable high cut-off at >25% FoxP3 which was considered many Treg was attributed in the study. Also the study used only refractory or relapsed cases of cHL, which distinguishes it from other studies observed.

In our study there may be a few factors to consider when reviewing the results. As already discussed in the method section, the majority of the tumour material (n=323) was counted by the author on whole tumour sections, while part of the material (n=121) was counted by an experienced hematopathologist on TMA. Although the reproducibility proved to be very good using statistical measurements, you can’t rule out the possibility that individual differences may appear when there are two different researchers counting on the same material, however even the same person can’t get the exact same percentage counting the same tumour section several times. There may also be differences in counting part of the material on whole tumour sections while part of the material is counted on TMA. Although the principle is the same using the two methods, counting only in fields with visible HRS, avoiding fibrosis and areas with normal biological structure, meaning the difference between the two methods are limited. An advantage with our study is the size of the study

Regulatory T cells in Hodgkin’s Lymphoma
which is notably larger than all previous studies on the subject. To more fully investigate how the number of Treg cells relates to clinical characteristics collection of clinical data for the Danish patient group is needed; especially important is this in survival analyses. This is currently in process and will hopefully result in future studies on the subject. It would also be interesting to count CTL on the same material (as conducted in several other studies) to see if there is correlation between CTL and Treg cells, and if this affects prognosis in the patient group. This is planned to be performed by the author in the future, and this taken together will hopefully result in material which is sufficient to publish in a scientific magazine.

In summary, all studies differ in their way of counting Treg cells, as already noted almost every study use a different antibody, and there is yet no consensus how to interpret the results of the role of Treg cells in HL.

Acknowledgements
My biggest and most sincere thank you to my main supervisor Ingrid Glimelius, for chasing away all my fears and worries about research, also for being so encouraging and always finding my results to be interesting. Thank you Gunilla Enblad for introducing me to the project in the first place. Thank you Rose-Marie Amini for showing me how to count Treg cells and CTL. Thank you Daniel Molin for helping me to update the survival database, and the help with statistica.

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Regulatory T cells in Hodgkin’s Lymphoma


