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Hodgkin Lymphoma in children, adolescents and young adults

ANNIKA ENGLUND

ACTA UNIVERSITATIS UPSALIENSIS UPPSALA 2017

ISSN 1651-6206
ISBN 978-91-554-9851-1
urn:nbn:se:uu:diva-316796

Dissertation presented at Uppsala University to be publicly examined in Rosénsalen, Ingång 95/96 Akademiska sjukhuset, Uppsala, Friday, 5 May 2017 at 09:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish. Faculty examiner: Associate professor Ingrid Øra (Lund University).

Abstract
Englund, A. 2017. Hodgkin Lymphoma in children, adolescents and young adults. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1314. 67 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-9851-1.

Hodgkin lymphoma (HL) is a heterogeneous condition varying from engaging one single lymph node site to a widespread condition. The prognosis with contemporary treatment is excellent for the vast majority. However, the treatment might cause severe late adverse effects in a proportion of the affected individuals.

We evaluated all children and adolescents diagnosed in Sweden and registered in the Swedish Childhood Cancer Register over a period of 25 years. The incidence has been stable and the overall survival (OS) is very good, comparable to the best results in the world. Approximately ten percent encountered a relapse, but even after relapse the chances of survival were good. During the study period there were no detectable changes in survival estimates. The use of radiotherapy has decreased.

Epstein Barr virus (EBV) and numbers of eosinophils, mast cells and macrophages in the tumors were investigated in 98 cases. Young children were more likely to express EBV. In patients with advanced disease the mast cell and macrophage counts were higher and they also had more affected laboratory parameters. Patients with Nodular Lymphocyte Predominant Hodgkin Lymphoma did not express EBV in the tumor, had significantly lower numbers of eosinophils, mast cells and macrophages and less affected laboratory parameters compared to classical HL.

Outcome and clinical presentation were investigated in a cohort of children, adolescents and young adults in Sweden and Denmark and treatment in pediatric and adult departments was compared. OS and event-free survival (EFS) did not differ between the three age groups nor between pediatric and adult treatment. However, the Danish pediatric patients had lower EFS, which corresponded to less use of radiotherapy. Adolescents and young adults shared similar characteristics, while children presented differently with less advanced disease and male preponderance.

Hospitalization rates and outpatient visits after end of treatment were evaluated to see whether the excess need of resources described in the literature is evenly distributed among the survivors or whether it is limited to a smaller group. Most of the patients had a low burden of health care use and the relapsing patients were the main drivers of the excess need.

Keywords: Hodgkin, pediatric, adolescent, young adults, microenvironment, eosinophils, mast cells, macrophages, Sweden, late adverse effects

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ISSN 1651-6206
ISBN 978-91-554-9851-1
urn:nbn:se:uu:diva-316796 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-316796)

Front cover:
Hodgkin Reed Sternberg cell. Aquarelle painting by Rebecka Englund.

List of Papers

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

I Englund A, Hopstadius, C, Enblad G, Gustafsson G, Ljungman G (2015). Hodgkin lymphoma - a survey of children and adolescents treated in Sweden 1985-2009. *Acta Oncol Jan;54 (1):41-8*.

II Englund A, Molin D, Enblad G, Karlén J, Glimelius I, Ljungman G, Amini RM (2016) The role of tumour-infiltrating eosinophils, mast cells and macrophages in Classical and Nodular Lymphocyte Predominant Hodgkin Lymphoma in children. *Eur J Haematol 97 (430–438)*.

III Englund A*, Glimelius I*, Rostgaard K, Ekström Smedby K, Eloranta S, Molin D, Kuusk T, de Nully Brown P, Kamper P, Hjalgrim H, Ljungman G*, Hjalgrim L* (2017) Hodgkin lymphoma in children, adolescents and young adults - a comparative study of clinical presentation and treatment outcome. * Authors contributed equally. Manuscript submitted.

IV Englund A*, Glimelius I*, Rostgaard K, Ekström Smedby K, Eloranta S, de Nully Brown P, Johansen C, Kamper P, Ljungman G, Hjalgrim H*, Hjalgrim L* (2017) Late adverse effects in children, adolescents and young adults in relapsing and non-relapsing patients with Hodgkin lymphoma. * Authors contributed equally. Manuscript.

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Abbreviations

ABVD	adriamycin, bleomycin, vincristine, dacarbazine
ASCT	Autologous Stem Cell Transplantation
BEACOPP	bleomycin, etoposide, adriamycin, cyclophosphamide, vincristine, prednisone, procarbazine
CAR	Chimeric Antigen Receptor
cHL	classical HL
CCL	Chemokine motif Ligand
CD	Cluster of Differentiation
CI	Confidence Interval
cHL	Classical Hodgkin Lymphoma
CHOP	cyclophosphamide, doxorubicin, vincristine, prednisone
COPDAC	cyclophosphamide, vincristine, prednisone, dacarbazine
COPP	cyclophosphamide, vincristine, prednisone, procarbazine
CTL	Cytotoxic T Lymphocyte
CTLA	Cytotoxic T Lymphocyte associated Antigen
CVP	cyclophosphamide, vinblastine, prednisone
DC	Dendritic Cells
DFS	Disease Free Survival
EBER	Epstein Barr Encoding Region
EBNA	Epstein Barr Nuclear Antigen
EBV	Epstein Barr Virus
ECP	Eosinophilic Cationic Protein
EuroNet PHL	Euro-Net Paediatric Hodgkin's Lymphoma
FDA	Food and Drug Administration
FDG-PET	Fluoro-Deoxy-Glucose Positron Emission Tomography
GPOH	German Pediatric Oncology and Hematology Group
GVHD	Graft Versus Host Disease
HDAC	Histone Deacetylase
HL	Hodgkin Lymphoma
HLA	Human Leucocyte Antigen
HPF	High Power Fields
HR	Hazard Ratio
HRS	Hodgkin Reed Sternberg
Ig	Immunoglobulin
IL	Interleukin
JAK	Janus Kinase

NHL	Non-Hodgkin Lymphoma
NOPHO	Nordic Society of Pediatric Hematology and Oncology
L	Ligand
LAG	Lymphocyte-Activation Gene
LD cHL	Lymphocyte Depleted cHL
L&H	Lymphocytic and Histiocytic
LMP	Latent Membrane Protein
LP	Lymphocyte Predominant
LR cHL	Lymphocyte Rich cHL
MC cHL	Mixed Cellularity cHL
MHC	Major Histocompability Complex
MMAE	MonoMethyl Auristatin E
mTOR	mechanistic Target Of Rapamycin
MOPP	mechlorethamine, vincristine, prednisone, procarbazine
NF-κB	Nuclear Factor kappa light-chain-enhancer of activated B cells
NK	Natural Killer
NLPHL	Nodular Lymphocyte Predominant HL
NOPHO	Nordic Society of Pediatric Hematology and Oncology
NS cHL	Nodular Sclerosis cHL
OEPA	vincristine, prednisone, etoposide, doxorubicine
OPPA	vincristine, prednisone, procarbazine, doxorubicine
OS	Overall Survival
PAX	Paired Box protein
PD	Programmed cell Death
PFS	Progression Free Survival
TNF	Tumor Necrosis Factor
T_H	Helper T cell
T_{Regs}	Regulatory T cells
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
SIOP	International Society of Paediatric Oncology
STAT	Signal Transducer and Activators of Transcription
WHO	World Health Organization

Introduction

Pediatric Oncology and Hematology

Treatment results of malignancies among children and adolescents have evolved substantially over the recent decades and are often described as a remarkable success, with substantial progress in estimated five-year overall survival (OS) in all tumor groups as illustrated in *Figure 1*.

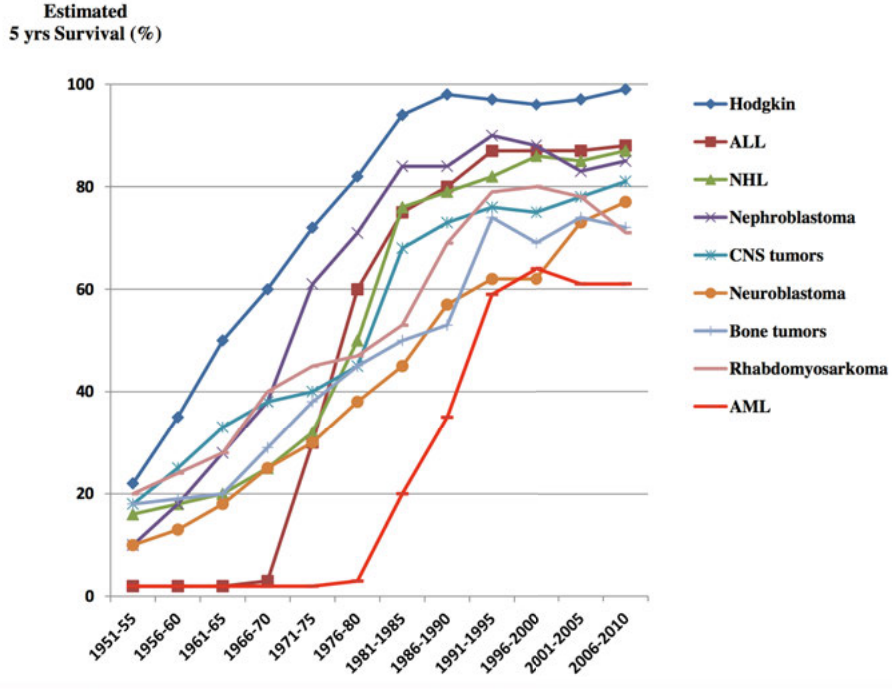

Figure 1. Five-year estimated overall survival in Sweden 1951-2010 (reproduced from Gustafsson et al [1] with permission).

Despite this progress and the fact that only about 300 children each year in Sweden are diagnosed with a malignant disease, malignancy is still the second most common cause of death in children 1-19 years of age (*Figure 2*).

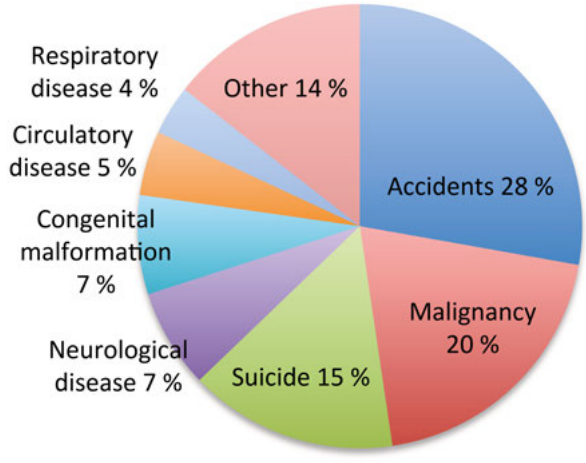

Figure 2. Causes of death among the 154 children and adolescents 1-19 years of age deceased in Sweden 2015 (picture created based on statistics from www.socialstyrelsen.se [2])

Diagnosis of pediatric malignant diseases is in Sweden centralized to six pediatric oncology and hematology centers (Göteborg, Linköping, Lund, Stockholm, Umeå, Uppsala), where the treatment is given and from which it is directed. Part of the treatment is often received at the local hospital in collaboration with one of the centers. All centers in Sweden use the same treatment protocols and representatives from each center meet regularly to discuss treatment and clinical challenges. Sweden is a member of the Nordic Society of Pediatric Hematology and Oncology (NOPHO), which is a network among the Nordic countries founded in 1980 as a platform for collaboration in clinical issues, research and education. More recently, Estonia and Lithuania joined the network. There is also an international network - The International Society of Paediatric Oncology (SIOP) - a global organization for collaboration to increase knowledge and improve treatment worldwide.

Hodgkin lymphoma

Hodgkin lymphoma (HL) is a disease originating from the lymph nodes, with a heterogeneous appearance, spanning from engaging a single lymph node site without any other signs or symptoms of disease, to a widespread condition with systemic symptoms and extra nodal involvement. It typically afflicts adolescents and young adults, but individuals of all ages may be affected. The pathology is peculiar, with only a few tumor cells surrounded

by a massive infiltrate of inflammatory cells. The microenvironment has attracted much attention in recent years, formerly regarded as "innocent" surrounding cells. With increasing evidence in the literature it is now considered as " a partner in crime" with the tumor cells, with an intricate interaction through different cytokines and signaling pathways.

With contemporary treatment the majority of the HL patients is cured (*Figure 1*), but a considerable proportion suffer from late adverse effects from the treatment, sometimes severely affecting daily life and exceeding the lymphoma as cause of death. The challenge today is to be able to reduce the treatment given, without hazarding the survival (*Figure 3*).

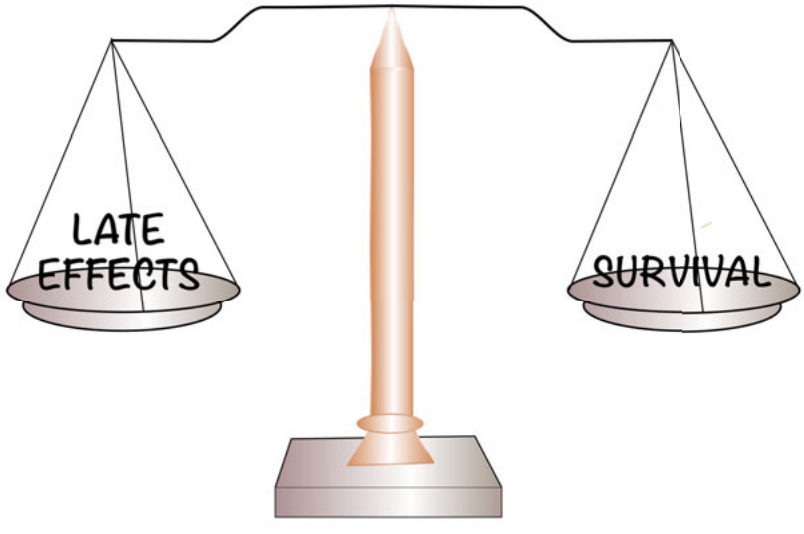

Figure 3. The challenge in treating patients with Hodgkin lymphoma is to find a balance between giving enough treatment to cure the disease, but without severe late effects.

History

In 1832, in the publication "On some Morbid appearances of the Absorbent Glands and Spleen" [3], the disease was described by Thomas Hodgkin, pathologist at Guy's Hospital in London. In his paper he presented seven cases with enlarged lymph nodes, in six cases in combination with spleen enlargement, without signs of infection or inflammation. Thomas Hodgkin is generally considered to be the one who first described the disease, although he, in his publication, refers to Marcello Malpighi (1628-1694) who, already in 1666 in *De Viscerum Structura* describes a young girl with enlarged spleen and lymph nodes resembling the condition.

Samuel Wilks [4] later named the condition Hodgkin´s disease. Dorothy Reed and Carl Sternberg then independently described the tumor cells in 1898 and 1902 [5, 6], and they have given the name to the pathognomonic tumor cell of classical Hodgkin lymphoma, the Hodgkin Reed Sternberg cell. In fact, later microscopic examination of the tissue (preserved in fixative for 97 years) was performed in 1926 by Fox [7] from the cases described by Thomas Hodgkin. He concluded that only three to four of the cases were HL and the remaining cases were either chronic inflammation due to syphilis or tuberculosis, or non-Hodgkin lymphoma (NHL). Hodgkin´s disease is today mostly referred to as HL [8].

Classification

The World Health Organization (WHO) classification of HL [9] is based on morphological findings with two main groups defined: classical HL (cHL) and nodular lymphocyte predominant HL (NLPHL), which is a subclass of HL with somewhat different morphology and biology. Classical HL is further divided into four subgroups: nodular sclerosis (NS cHL), mixed cellularity (MC cHL), lymphocyte rich (LR cHL), and lymphocyte depleted (LD cHL).

Pathological characteristics

The cHL tumor is characterized by very few tumor cells surrounded by a large mass of inflammatory cells such as fibroblasts, lymphocytes, neutrophils, eosinophils, mast cells and macrophages. The tumor cells in cHL, the Hodgkin Reed-Sternberg (HRS) cells, are pathognomonic for cHL and the HRS clones consists of a mixture of mononucleated Hodgkin cells and binucleated Reed Sternberg cells (*Figure 4*).

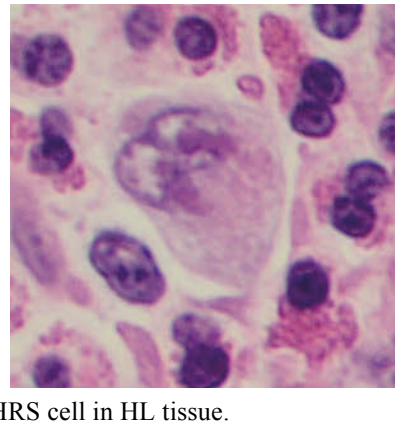

Figure 4. Binucleated HRS cell in HL tissue.

The tumor cells have been shown, in most cases, to be of B cell origin [10-12], indicated by detection of somatically mutated monoclonal immunoglobulin gene rearrangements, and considered as germinal center or post-germinal center derived. However, the HRS cells rarely express B cell antigens, probably due to loss of B cell specific transcription factors and/or epigenetic alterations of B cell specific genes [13-16]. Characteristic for the HRS cells is the expression of Cluster of Differentiation (CD) 30, a receptor that is a member of the TNF-receptor superfamily. It also most often positive for CD15 and for paired box protein 5 (PAX5), whereas the B cell marker CD20 is usually negative. There are, however, also cases with expression of T cell markers with T cell receptor gene rearrangements and lack of immunoglobulin (Ig) gene rearrangements [17, 18]. Normally, B cells with unfavorable mutations in the B cell receptor are selected for apoptosis in the germinal center. HRS cells escape apoptosis through different anti-apoptotic signals. The Nuclear Factor kappa-light chain enhancer of B cells (NF-κB) pathway and the Janus Kinase/Signal Transducer and Activators of Transcription (JAK/STAT) pathway are constitutively active in the HRS cells [19, 20], and help the cells to survive. Different mechanisms most likely contribute to the activation of these pathways, such as mutations and through signals via cytokine receptors, tyrosine kinases, the TNF super family and also Epstein Barr (EBV) infection (described below).

The tumor cells in NLPHL, the lymphocyte predominant (LP) cells or Lymphocytic and Histiocytic (L&H) cells still express B cell markers, indicating their B cell origin [21-24].

Microenvironment

The microenvironment in HL, formerly considered as “innocent” surrounding, has gained much attention in research during the recent decades, and is now considered to be of importance for the development of the tumor, and for the prognosis. The surrounding cells, as described above, communicate with the tumor cells via cytokines and different signaling pathways, summarized by Küppers [20].

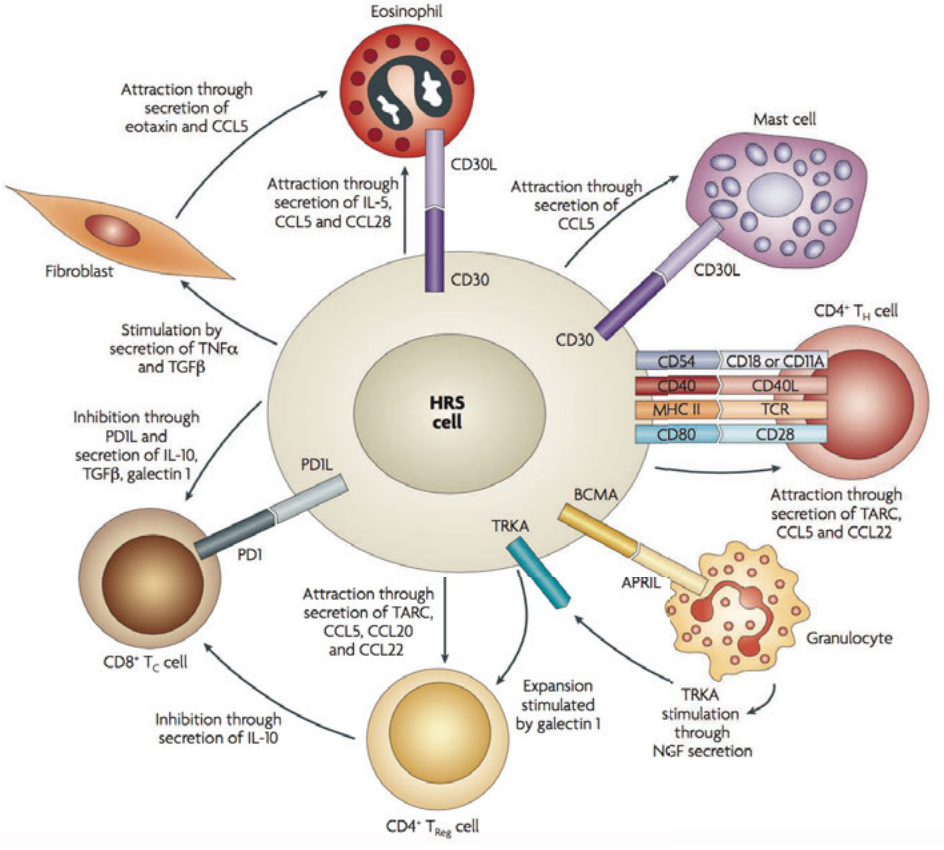

Figure 5. Overview of the complex interactions in the microenvironment in HL (picture from Küppers [20] with permission).

Eosinophils

Eosinophil granulocytes are a part of the normal immune system and are derived from a pluripotent stem cell in the bone marrow [25]. They are predominantly found in the gastrointestinal tract [26], but also migrate to the thymus, the mammary gland and the uterus [27]. They are measurable, in low numbers, in peripheral blood. Their role has been described as a part of the defense against parasitic infections [28], in the development of asthma and allergic diseases [29] and in gastrointestinal diseases [30].

CD30, expressed by the HRS cells, can be stimulated via the CD30 ligand (CD30L), expressed by eosinophils and mast cells and has been shown to enhance proliferation of cHL cell lines [31, 32]. Some HL cases present with large numbers of eosinophils in the tumor. Eosinophil rich tumors have been described with poorer survival [33, 34], but also with no significant difference [35, 36]. The function of the eosinophils is mediated through degranulation and release of the contents of the granules that contain

different proteins, e. g. eosinophilic cationic protein (ECP). ECP levels have in serum have been correlated to higher numbers of eosinophils and to negative prognostic factors [37]. In a cohort of HL, self-reported history of asthma was correlated to high numbers of eosinophils in the tumor and to the ECP genotype ECP434GG [38]. In pediatric HL eosinophilia has been associated to extra nodal disease, but not to worse prognosis [39].

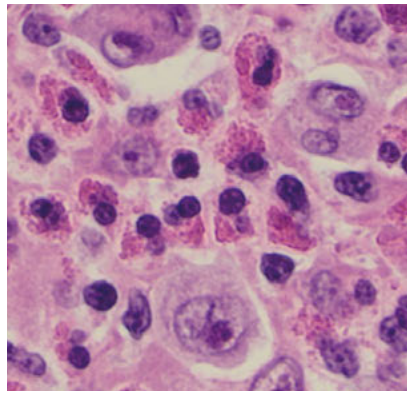

Figure 6. Eosinophils (Hematoxylin-eosin) in HL tissue.

Mast cells

Mast cells originate from the bone marrow as mast cell progenitors and mature in peripheral tissue. They are thought to derive from granulocyte/ monocyte progenitors [40]. Mast cells are present in all tissues in the human body, but most prominently in the skin, the airways and the gastrointestinal tract. They play an important role in allergy, asthma and autoimmune disorders, but are also thought to take part in the immunological host defense against infections [41]. Mast cells are described to affect tumor development, both promoting and protecting [42]. The way of action is thought to depend on the type of tumor and host conditions.

Mast cells have been associated to poorer disease-free survival (DFS), higher white blood cell count and lower hemoglobin rate in HL in adults [43], analyzed with an antibody recognizing tryptase. These findings have been confirmed in a later study [44] examining factors for primary refractory or early relapsed cases, staining for c-kit/CD117 to recognize mast cells. The mast cells express CD30L and may contribute to the stimulation of HRS through the binding to CD30 [31, 32, 45]. In HL tumors mast cells are the predominant CD30L positive cells [31]. Mast cells may be recruited by the Chemokine motif Ligand (CCL5)/Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), produced by the HRS cells [46]. In pediatric HL the role of mast cell infiltration has not been elucidated.

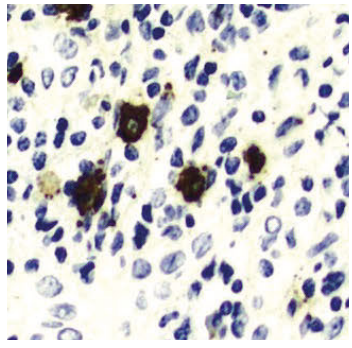

Figure 7. Mast cells (Tryptase staining) in HL tissue.

Macrophages

Macrophages have for a long time been an established part of the immune system. They were already discovered at the beginning of the 20th century by Metchnikoff [47], a Russian scientist who received the Nobel Prize in 1908 for his description of cells he named phagocytes as a part of the host defense against infection. Most of the macrophages originate from bone marrow precursor cells developing into monocytes that circulate in the peripheral blood and turn into macrophages or dendritic cells (DC) when migrating into connective tissue. In the target tissue they develop to different subtypes of macrophages. The macrophages protect the host by phagocytosis of microbes and presenting antigens to T cells and B cells and play an important role in tissue hemostasis and remodeling. Cytokines produced by the macrophages and the surrounding cells regulate the function of the macrophage [48]. It has been suggested that the differentiation of the macrophages results in two different phenotypes, classically activated M1 and the alternatively activated M2 [49]. M1 macrophages are described as being strongly microbicidal and tumor suppressive, M2 immunosuppressive and possibly tumor promoting and most of the tumor-associated macrophages are of the M2 type [50]. Recently however, it has been suggested that his classification is in a need of revision [51], taking into account that the macrophages, rather than forming stable subsets, respond to and interact with their environment (cytokines, surrounding cells) to form more complex phenotypes.

Macrophages are present in HL tumors and have, in several studies on adults, been associated with worse prognosis, but also with no significant difference in outcome. The main markers used are CD68 and CD163. Guo et al [52] have included 22 of these studies in a meta analysis and concluded that high numbers of either CD68+ or CD163+ macrophages is a predictor of worse outcome, both OS and PFS. In children, only a few studies have been performed, with contradicting results on survival estimates. Barros et al [53] studied 100 cases of HL from a developing area in Brazil and in this material progression-free survival (PFS) was lower in cases with high numbers of

CD163+ cells, but high numbers of CD68+ cells did not affect OS or PFS. Gupta et al [54] presented a treatment-failure enriched cohort of 96 patients where no association between OS or event-free survival (EFS) could be found in macrophages evaluated with either CD163 or CD68. High rates of HRS cells in the tumors were associated with worse EFS in univariate analysis, but did not reach significance in the multivariate analysis. Zameer et al. 2015 [55], presented a cohort from India, where they demonstrated a large proportion of Epstein Barr Virus (EBV) positive cases (93 % in total, <10 years 97.3 %, 10-15 years 83.7 %, (measured with EBER (Epstein Barr encoding region) in situ hybridization) and many cases presented with high macrophage counts, detected with CD68 (86 % >25 %, none < 5 %).

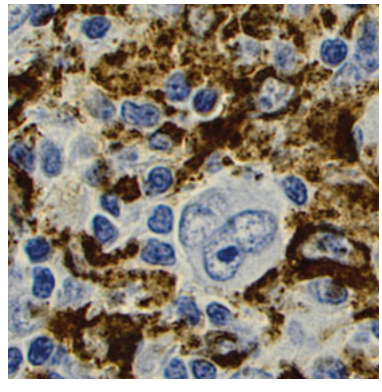

Figure 8. Macrophages (CD68 staining) in HL tissue.

T cells and NK (natural killer) cells

T cell progenitors originate from stem cells in the bone marrow and then undergo further maturation in the thymus [56]. The most abundant cells around the HRS cells are T cells and they are a mixture of CD8+ cytotoxic T cells (CTL), CD4+ T helper cells (T_H) and CD4+ regulatory T cells (T_{Regs}) with the two latter being the most frequent [57]. The T cells form rosettes around the HRS cells [58] by aggregating around the tumor cells, mediated by secretion of cytokines by the HRS cells to attract the T cells and by several interactions through adhesion molecules on the surface of the cells [59]. The CD40-CD40L binding activates the NF-κB pathway, which in HL stimulates the HRS cells [60].

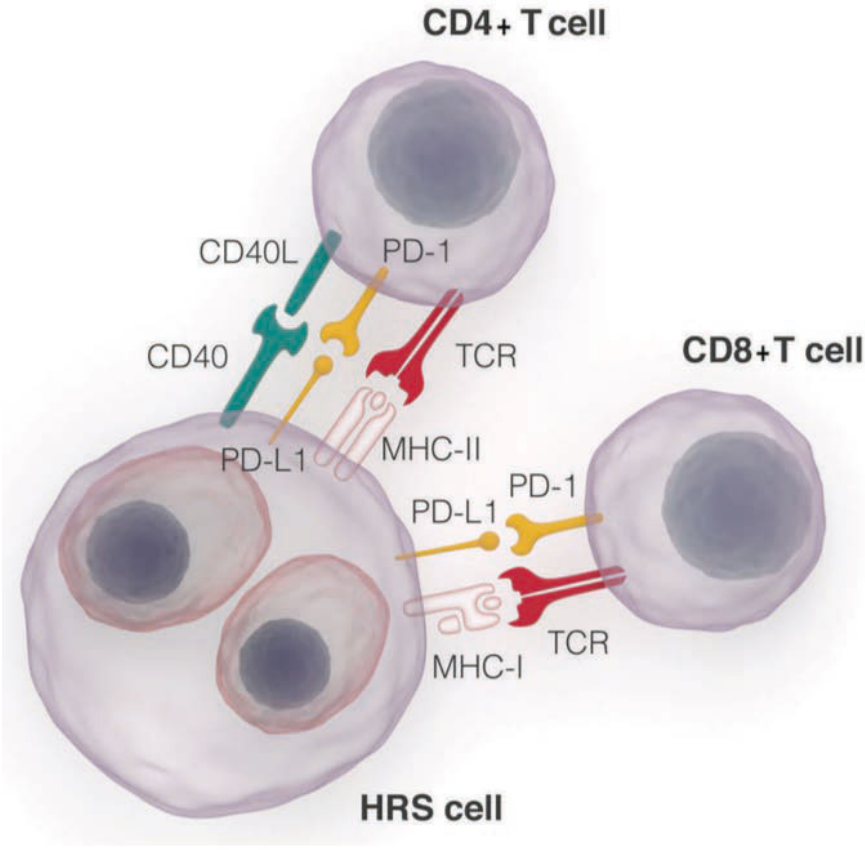

Figure 9. Interactions between the HRS cell and the T cells. (picture from Vardhana et al [61] with permission).

Activation of the T cells is mediated through antigen recognition on MHC molecules on the surface of antigen presenting cells (APC) in combination with a co-stimulatory signal by binding to CD28 on the T cell and receptors for different cytokines [62].

One of the main missions for cytotoxic lymphocytes (CTLs and NK cells) is to protect the body from foreign attack; such from bacteria, viruses and tumors and by recognizing them killing and eliminating those cells. The HRS cells have several ways of escaping these attacks, where the rosette formation of the CD4+ cells acts as one protecting shield. The T_{Regs} are also thought to play an important functional role [63], by the inhibition of the CTLs through the expression of cytotoxic T-lymphocyte associated protein 4 (CTLA4), secretion of Interleukin (IL) 10, expression of Programmed cell Death (PD)-1 and potentially also other mechanisms [57, 63, 64]. Further, Major Histocompatibility Complex (MHC) molecules are often down modulated in the HRS cells, thus presenting another way for the tumor cells

to escape the immune system since the presence of MHC is required for antigen recognition [65-67]. The expression of Fas Ligand/CD95 that has been described in the HRS cells may also induce apoptosis of the activated T cells [68, 69].

Programmed Cell Death 1 and its ligands

Programmed Cell Death 1 (PD-1) is a member of the CD28 co-stimulatory receptor superfamily and can be expressed on the surface of several cells in the immune system, such as B cells, T cells, NK cells, activated monocytes and dendritic cells [70]. The binding to its ligands PD-L1 and PD-L2 acts as an inhibitory signal to T cells in combination with signaling through the T cell receptor [71]. The genes for PD-L1 and PD-L2 are located on chromosome 9p24.1 [70]. The expression of the ligands of PD-1 by the HRS cells is thus one way of escaping the immune system, thereby inactivating the T cells. Genetic alterations in this region with gain of gene copies of 9p24.1 have been associated with increased PD-L1 expression by the HRS cells, more unfavorable stage and lower PFS [72]. Increased levels of PD-1 positive lymphocytes in the tumor have been associated with worse OS [73]. The expression of PD-1L may also be increased by EBV infection [74].

The PD-1/PD-1L checkpoint is one of the most interesting targets for the development of new treatment options (described further in "Novel treatment approaches").

Epidemiology

The epidemiology of Hodgkin's lymphoma is peculiar, with two age peaks [75-77] that vary in different populations. The pattern seems to change with the socioeconomic status of the society. In developing countries the early peak occurs in childhood whereas in societies with a high socio-economic standard the young age peak is about at 25 years of age (*Figure 10*). There is also a pattern in between, described in Asia, with a transition from a high incidence in childhood to a high incidence in early adulthood in parallel with economic development [78]. Recently, a third peak in affluent settings has been described in young children [79].

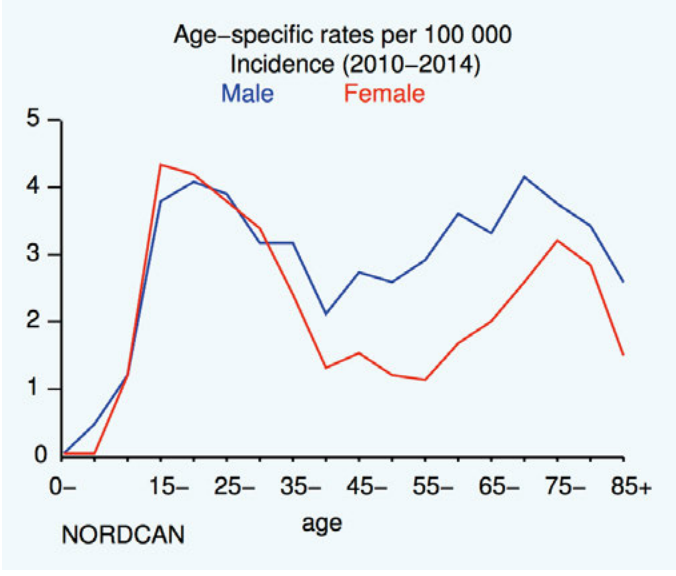

Figure 10. Incidence in the Nordic countries 2010-2014, illustrating the two main peaks. (picture from Engholm et al [80] with permission).

There have been two major hypotheses in HL epidemiological research, the "the multiple diseases" model and the "late infection" model. Briefly, the first hypothesis suggest the different age peaks to represent etiologically distinct entities, with three distinguished age periods (0-14, 15-34 and over 50 years) [75, 76] and that he tumors in the young adult group (15-34 years) are caused by chronic inflammation due to infection [75]. The "late infection" model [77, 81, 82] focuses on children and young adults and the theory that HL is due to a common childhood infection, with different onset in different socioeconomic settings. Earlier studies have described a relationship of decreased risk for HL in young adults with factors associated with higher infectious pressure in childhood, such as larger sib-ship size [83-85], especially number of older siblings [86], although other studies have shown no association [87, 88] to the number of siblings. However, as the socioeconomic factors and status in the community changes, the size of the sib-ship might have less significance and attending to day care may as a measure for early exposure to infections be more adequate, as suggested by Chang et al [89].

Epstein-Barr virus and infectious mononucleosis

EBV is a lymphotropic virus that has been widely described in the literature with association to HL and has been concluded to be causal, at least in EBV-positive cases [90]. EBV can be detected in HRS cells and has been shown

to be monoclonal [91], which indicates that the infection precedes the malignant transformation. Furthermore, the risk of HL is increased after infectious mononucleosis, described in several studies, reviewed by Hjalgrim [92]. The risk has been shown to be restricted to EBV-positive HL with an odds ratio (OR) of 3.96 (95 % CI 2.19-7.18) and most pronounced for younger adults 18-44 years of age and occurred after a median of 2.9 (1.8-4.9) years [93]. This study did however not include individuals younger than 18 years of age. An increased risk has been described up to at least ten years after mononucleosis [94, 95].

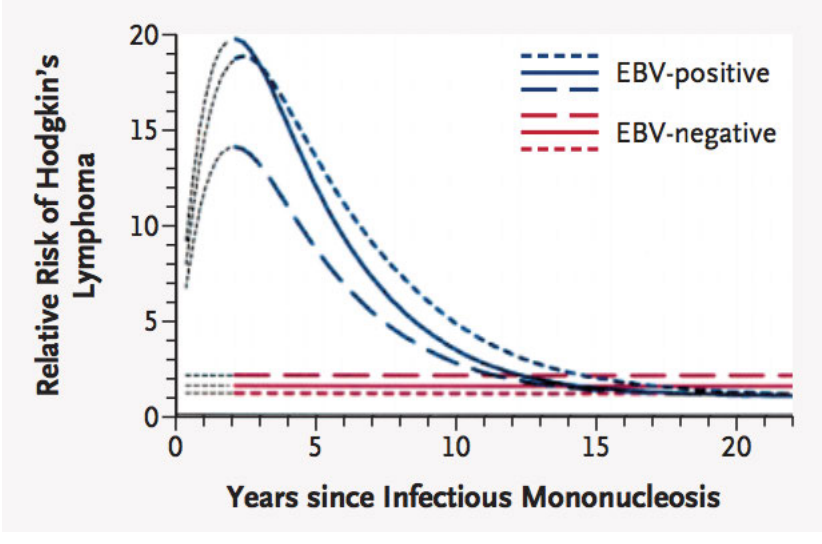

Figure 11. The risk of HL after infectious mononucleosis is restricted to EBV-positive Hodgkin. Reproduced from Hjalgrim et al [94], Copyright Massachusetts Medical Society with permission.

EBV-positive HRS cells express virus proteins, Latent Membrane Protein 1 and 2A (LMP), and EBNA1 (EBV Nuclear Antigen) [20, 96, 97]. LMP1 is an oncogene that can aggregate in the cell membrane and mimic an activated CD40 receptor, which will activate the NF-κB pathway and contribute to the survival of the HRS cell [98]. LMP2A may help the cell to survive by acting as a B cell receptor [99, 100] and by escaping the immune system [101]. EBNA1 is present in all EBV-infected cells, being a part of the viral replication and has also been shown to up regulate CCL20 and thereby attract regulatory T cells to the tumor [102]. EBNA1 also down regulates a putative tumor suppression gene, protein-tyrosine phosphatase-κ [103].

However, not all HL cases are EBV-positive. The overall frequency of EBV-positivity is around 40 % but varies with age, sex, histological subtype and ethnic origin [104]. The etiology of EBV-negative HL is not known. A theory of "hit and run", i.e. EBV would in fact be the causative agent, but

then “escapes”/is eliminated by the immune system, has been discussed [105-107], but neither proven nor excluded.

Genetic susceptibility

There is an increased risk among first-degree relatives to be afflicted by HL, recently investigated in a large Nordic cohort [108], where the highest risk were among siblings (6.0-fold (95 % CI 4.8-7.4), especially same sex twins (57-fold (95 % CI 21-125)) and for those with more than one affected first-degree relative (13-fold (95 % CI 2.8-39)).

Genetic association studies have revealed an association to the Human Leucocyte Antigen (HLA) locus on chromosome 6, where different HLA alleles have been shown to be either protective or risk associated, reviewed by Kuschekhar et al [109]. HLA is a molecule expressed on the cell surface presenting pathogen or tumor-derived peptides to T cells to activate the host defense. There are two main HLA molecules. HLA class I (HLA-A, HLA-B and HLA-C) is expressed on almost all somatic cells and present endogenous peptides to cytotoxic T-lymphocytes (CD8+). HLA class II (HLA-DP, HLA-DQ and HLA-DR) is expressed by antigen presenting cells (i e macrophages, monocytes, dendritic cells) and present exogenous peptides to CD4+ T cells.

Ethnicity should be taken into consideration in these studies since HLA alleles differ between populations leading to possible differences in the risk associations. With regard to HLA, the EBV status of the tumor is also of interest, where the associations differ between EBV-positive and EBV-negative HL. EBV-positive HL is mainly associated with different subtypes of HLA class I. In Caucasians HLA-A*01 has been associated with increased risk in EBV-positive HL and the allele HLA-A*02 with decreased risk [110-113]. EBV-negative HL has been described mainly associated with alterations in the HLA class II [109].

Clinical presentation

The presentation of HL is generally with enlarged lymph nodes in one or several regions, and, in a proportion of cases, with B-symptoms. B-symptoms are defined as weight loss (> 10 % of the total body weight over a time less than six months), profuse night sweats and unexplained and persisted fever (more than 38 degrees).

The most common sites are on the neck and in the mediastinum, but all lymph node regions could be engaged. The disease also sometimes spreads to extra nodal sites, such as lung, liver, bone marrow or bone.

Depending on the number of regions engaged, the disease is divided in different stages, according to the Cotswolds modified version of the Ann Arbor staging classification [114], table 1.

Table 1. *Stage classification*

Stage	Engaged sites
I	Only one lymph node region
II	Two or more regions on the same side of the diaphragm
III	Two or more regions on both sides of the diaphragm
IV	Engagement of extra nodal sites beyond ”E-sites” (see below). Liver or bone marrow engagement is always classified as stage IV.

E-sites (referred to as E): engagement of a single extra nodal site near or adjacent to a known engaged lymph node site. A/B: absence/presence of B-symptoms.

Treatment

cHL

Adults with cHL in Sweden are uniformly treated in their respective clinics according to national guidelines [115], briefly with two to four courses of ABVD (doxorubicin, bleomycin, vincristine, dacarbazine) followed by 20-30 Gy radiotherapy in stage I-IIA and six to eight courses of ABVD or BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, prednisone, procarbazine) to stage IIB-IV depending on risk factors. FDG-PET after two courses of chemotherapy is used to escalate or de-escalate treatment in advanced stages. Radiotherapy is not used routinely in advanced stages.

From 1996, all pediatric patients with HL have been treated according to the German Pediatric Oncology and Hematology Group (GPOH), protocols and from 2006, those of the European Euro-Net Paediatric Hodgkin’s Lymphoma Group. Patients have been included in the studies when they have been open and the patient/family has agreed to participate. In these protocols, the patients have been divided into therapy groups (TG).

TG 1: Stage IA/B and II A
TG 2: Stage I A/B_E, IIA and III A
TG-3: Stage IIB_E, IIIA/B_E, III B and IVA/B

Before 1996 MOPP (mechlorethamine, vincristin, prednisone, procarbazine) (or MOPP/ABVD) were used in 4–8 cycles followed by RT involved field (IF) 25–40 Gy or extended field (EF) (mantle field, inverted Y-field or total nodal radiotherapy) up to 40 Gy depending on stage and response to therapy. Since 1996 different combinations of OPPA/OEPA (vincristine, prednisone, procarbazine vs. etoposide, doxorubicine)/COPP (cyclophosphamide, vincristine, prednisone, procarbazine) have been used, and since 2006 OEPA/COPP/COPDAC (procarbazine replaced by dacarbazine) has been used according to the protocols of the different clinical trials (GPOH-HD 95, GPOH-HD 2002 pilot, GPOH Interim, and EuroNet-PHL-C1) (table I). The doses of radiotherapy have been gradually reduced, and in some groups

omitted, depending on response to therapy measured by volume and/or FDG-PET (fluoro-deoxy-glucose positron emission tomography) uptake reduction. Tumor response after two courses measured by FDG-PET have been shown to predict outcome, and are used to determine whether to give radiotherapy or not [116-119].

Table 2. *Overview of different treatment protocols (reproduced from paper I [120] with permission).*

Protocol	**TG-1**	**TG-2**	**TG-3**
GPOH HD-95	F: 2 OPPA	F: 2 OEPA, 2 COPP	F: 2 OEPA, 4 COPP
	M: 2 OEPA	M: 2 OEPA, 2 COPP	M: 2 OEPA, 4 COPP
	RT: CR: No RT Non CR: 20 Gy IF+boost remaining tumour, max 35 Gy	RT: see TG-1	RT: see TG-1
GPOH HD 2002 pilot	F: 2 OPPA M: 2 OE*PA	F: 2 OPPA, 2 COPP M: 2 OE*PA, 2 COPDAC	F: 2 OPPA, 4 COPP M: 2 OE*PA, 4 COPDAC
	RT: See GPOH HD-95 TG-1.	RT: All 20 Gy IF+boost remaining tumour, max 35 Gy	RT: See GPOH HD 2002 pilot TG-2.
EuroNet PHL-C1	M/F: 2 OE*PA	M/F 2 OE*PA, 2 COPP or COPDAC (randomisation)	M/F 2 OE*PA, 4 COPP or COPDAC (randomisation
	RT: CR or non-CR PET neg: no RT. Others: 20 Gy IF + boost remaining tumour max 30 Gy.	RT: See EuroNet-PHL-C1 TG-1	RT: See EuroNet-PHL-C1 TG-1

CR=complete remission, RT= radio therapy, IF= involved field, *=25 % more etoposide.
OPPA= vincristine 1,5 mg/m^2 i.v. day 1, 8, 15; procarbazine 100 mg/ m^2 p.o. day 1-15; prednisone 60 mg/m^2 p.o. day 1-15, doxorubicine 40 mg/m^2 day 1, 15.
OEPA= vincristine 1,5 mg/m^2 iv day 1, 8, 15; etoposide 125 mg/m^2 i.v. day 1-4 (-5 in OE*PA); prednisone 60 mg/m^2 p.o. day 1-15, doxorubicine 40 mg/m^2 day 1, 15.
COPP= Cyclophosphamide 500 mg/m^2 day 1, 8; vincristine 1,5 mg/m^2 day 1, 8; prednisone 40 mg/m^2 day 1-15, procarbazine 100 mg/m^2 p.o. day 1-15.
COPDAC= procarbazine in COPP is replaced by dacarbazine i.v. 250 mg/m^2 day 1-3.

NLPHL

Historically patients with NLPHL were treated the same way as cHL patients, but with growing knowledge of the favorable prognosis in this group the treatment has been diminished. The expression of CD 20 in the tumor cells of NLPHL cases makes it suitable for treatment with rituximab

(Mabthera®), an antibody directed against CD 20. The use of rituximab has improved outcome for patients with NLPHL [121].

In adults the treatment recommendations for limited stages are four courses of rituximab followed by radiotherapy and if bulky disease three courses of rituximab in combination with CHOP and radiotherapy. In advanced stages eight courses of rituximab or six courses of rituximab and CHOP dependent on risk factors [115].

In children Sweden has decided to participate in the Euro Net-PHL-LP1 study of limited stages of NLPHL where the recommendations are resection alone if possible and then "wait and see". If resection is not possible three courses of CVP (cyclophosphamide, vinblastine, prednisone) is given. In more advanced stages there is no specific national guidelines for pediatric patients in Sweden and the treatment is often discussed with an adult oncologist since the patients are often approaching adult age.

Late effects of treatment

The treatment outcome in HL with contemporary protocols is excellent. However, the late effects due to treatment are considerable. The frequency of HL specific deaths declines after 10-15 years, but death from other causes, related to the treatment, continues to increase [122-125]. The main late effects due to the treatment are secondary malignancies [122, 123, 126-128], cardio-pulmonary diseases [129-135] (lung fibrosis, cardiac failure, valvular disease and arteriosclerosis), muscular atrophies [136], endocrinological effects such as infertility [137, 138], thyroid abnormalities and growth retardation [138, 139]. There are also several studies on fatigue, summarized by Daniëls et al [140].

Attempts are ongoing to minimize the therapy while maintaining the effect on survival. There are several aspects to consider when discussing late effects. Firstly, many retrospective studies report late effects due to outmoded treatment that was more toxic. Secondly, the risk of late effects is likely to be dependent on age at time of diagnosis and treatment. Thirdly, the frequency of those diseases in the normal population varies with age.

However, the knowledge of late effects from the earlier treatment regimens is important in the care of the survivors and in tailoring the treatment given today.

Secondary malignancies

The occurrence of secondary malignancies following treatment for HL were first described in the beginning of the 1970s [141, 142] and can be divided in three categories; leukemia, especially acute myeloid leukemia, NHL and solid tumors. The incidence of secondary leukemia is dependent on the amount of alkylating agents used [143-145]. The incidence of secondary myeloid leukemia has declined over time [146], most possibly reflecting

changes in treatment. The development of NHL after treatment for HL has been reported in excess rates [126, 127]. The reason for that could be debated. It could be a transition from HL to NHL, a late effect from therapy or possibly due to host factors, such as immunosuppression. Solid tumors represent, with long follow up since they typically occur after up to ten years, the major part of the secondary malignancies in HL [123, 126-128]. In a recent study declining incidence among female HL survivors was reported, possibly reflecting the efforts in decreasing the numbers of patients receiving radiotherapy [147].

Cardiovascular- and lung disease

There is a well-documented long-term risk of cardiac complications following treatment for HL, which can present in several ways, such as cardiomyopathy, coronary artery-, valvular and pericardial disease, and arrhythmias. The elevated risk has been directly linked to radiotherapy doses as described by Schellong et al. in a cohort treated in childhood or adolescence [130]. Chemotherapy is also related to an excess risk of of cardiac complications, mainly correlated to anthracycline use [148]. However, the results from a recent Swedish study on adults with HL [149] that reports continually decreasing mortality from diseases in the circulatory system from the 1980s and onwards are encouraging in the attempts on diminishing late effects from treatment.

The use of bleomycin is correlated to a risk of developing bleomycin-induced pneumonitis [132, 150] and is a part of the ABVD treatment. ABVD is no longer used as primary treatment in children and adolescents in Sweden, but is used in adults and may be considered in children with advanced disease or in second or third line treatment. Radiotherapy to the lungs may cause pneumonitis and lung fibrosis [133, 151].

Muscular atrophies

Muscular weakness and atrophy have been described mainly after mantle field radiotherapy, which was earlier the standard method for radiotherapy of cervical HL [136, 152-154]. This method is not used in contemporary protocols. However, there are a lot of HL survivors suffering from this condition, emphasizing the need for awareness among clinicians meeting these patients.

Endocrine effects

Van Dorp et al have reviewed a variety of articles on endocrinological effects after HL treatment [138].

Infertility

The risk of infertility after HL treatment is mainly linked to the use of alkylating agents and pelvic radiotherapy [138]. Procarbazine, earlier used in

pediatric protocols (see above), is known to be gonadotoxic [155] and correlated to male infertility. It has now been replaced by dacarbazine that has been shown to be equally effective [156]. The Euro Net CHL-1 study has investigated whether the change in therapy will result in less infertility. However, data from this study are not yet available. In a prospective study on HL female survivors treated during childhood or adolescence the chance of parenthood was not affected by accumulating doses of procarbazine or cyclophosphamide, but was affected by pelvic radiotherapy [157].

Thyroid dysfunction

Thyroid dysfunction is frequent after treatment for HL, with hypothyroidism being the most common diagnosis, but also hyperthyroidism, thyroid nodules and thyroid cancer occur. The occurrence of thyroid abnormalities has been linked to radiotherapy of the neck [158, 159], whereas chemotherapy alone did not affect the thyroid function [139].

Growth retardation

There are several explanations for reduced growth in cancer patients treated as children or adolescents, such as treatment related side effects (the treatment composition, infections, etc.), malnutrition, and the malignancy itself. Spinal radiotherapy in HL is correlated to lower final height [160, 161], whereas chemotherapy alone does not seem to have the same influence, although van Beek et al [139] have reported reduced height in males treated with MOPP without radiotherapy.

Fatigue

Several studies have reported significant levels of fatigue after treatment for HL [140]. In a recent study on quality of life, specifically addressing HL survivors treated in childhood [162] high scores were obtained for fatigue, especially for young women. The causality of fatigue in HL is not entirely understood and is probably multifactorial. Fatigue is present prior treatment, indicating that it is not only treatment related and may be a part of the disease presentation [163]. Daniëls et al [164] confirmed the high frequency of fatigue compared with a sex and age matched population and found a significant association between fatigue and anxiety or depression. However, fatigue was more common than depression/anxiety and a clear causal association cannot be assumed, since other comorbidities may also be of importance.

Novel treatment approaches

Combined modality treatment with conventional chemotherapy and radiotherapy has been the standard treatment for decades. The growing body of knowledge of the long-term late effects of these treatment modalities and in

a proportion of the patients' treatment failure has generated a need for more specific and tailored treatment regimens.

Before 2011, there had been no new approval of new drugs specifically for HL since 1977 [165]. However, the past decades of research, focusing on the microenvironment around the tumors cells, has been fruitful.

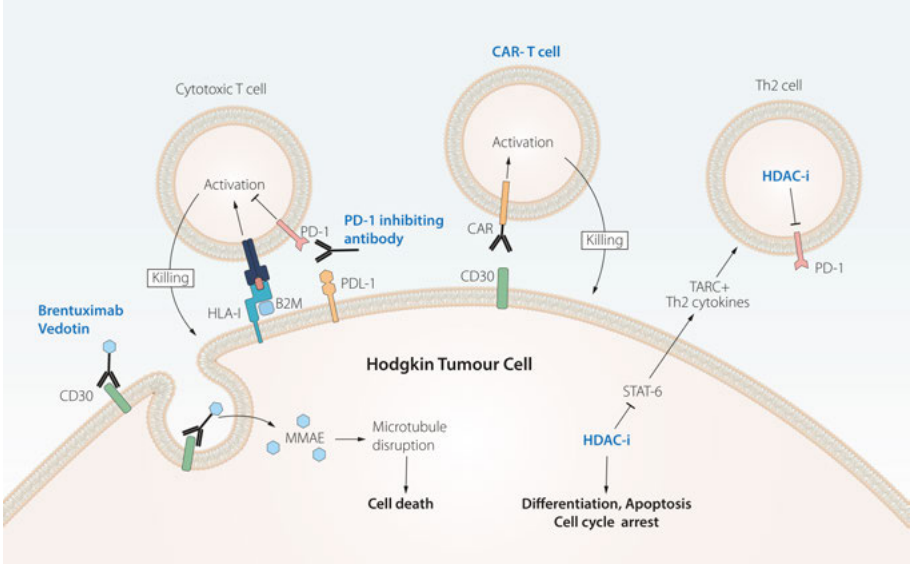

Figure 12. Overview of some interesting new therapeutic approaches (picture from Glimelius et al [165] with permission).

Brentuximab Vedotin

In 2011, brentuximab vedotin, an antibody against CD 30 and conjugated to a cytotoxic antitubulin agent, monomethyl auristatin E (MMAE) was approved by the US Food and Drug Administration (FDA) for treatment of patients not responding to conventional treatment. Glimelius and Diepstra [165] have reviewed published ongoing or recently closed trials with brentuximab vedotin in first or second line treatment, in consolidation after autologous stem cell transplantation (ASCT) or in relapse after ASCT. A phase I study reported promising results in 2010 [166] and was followed by the Phase II trial published in 2012 of 102 patients with refractory or relapsed disease had an overall response rate of 75 % (95 % CI 65-83 %) [167]. The five-year follow up study reported an OS of 41 % (31-51 %) [168]. There are now numerous trials investigating brentuximab vedotin in different settings and in combination with other treatments (clinicalTrials.gov). The most common treatment side effects are peripheral sensory neuropathy, nausea, fatigue, diarrhea, pyrexia, arthralgia, pruritus, myalgia, peripheral motor neuropathy and alopecia [167]. However, the potential late adverse effects remain to be further investigated.

Checkpoint inhibitors

Nivolumab and pembrolizumab are antibodies directed against PD-1, and may reactivate tumor-specific T cells and thus reinforcing the immune system to act on the tumor cells. PD-1 inhibitors have been shown to be effective in several cancer diagnoses, with a substantial effect on HL. In a phase I study, 23 patients with relapsed or refractory HL received were treated with nivolumab with an objective response of 87 % (17 % complete response, 70 % partial response) [169]. A phase II study is ongoing on patients previously treated with ASCT, brentuximab vedotin or both and a part of that study has been published with an overall response rate of 66 %, of which 8.8 % had a complete response [170]. The phase I study on pembrolizumab [171] had an overall response rate of 65 % of which 16 % had a complete response and is further investigated in a phase II trial (KeyNote 087). The most commonly reported side effects in HL patients are rash, decreased platelet and lymphocyte count, fatigue, pyrexia, diarrhea, nausea, pruritus, cough, pneumonitis and enteritis. However, most of the patients did not have to interrupt their treatment due to side effects [169, 171-174]. Previous allogeneic stem cell transplantation was initially considered to be a risk factor for graft versus host disease (GVHD) due to the reactivation of T cells and these patients were initially excluded from studies. Reports on results from treatment of patients relapsing after allogeneic stem cell transplantation are scarce but indicate that patients without active GVHD can be safely treated with PD-1 inhibitors [175-177].

How to best proceed with therapy after achieving tumor control with PD-1 blockade is still unknown. Issa et al[178] have suggested different options to choose from, depending on the patients status and how aggressive the lymphoma is. Their suggestions are to continue checkpoint blockade, cease therapy with potential re-treatment or to proceed to transplantation or treatment with chimeric antigen receptor T cell therapy (CAR-T, se below).

CAR T

One targeted therapy applies the use of chimeric antigen receptor T (CAR T) cells where T cells are designed to recognize tumor cells irrespective of the presentation on HLA-molecules. The receptor is composed of the signaling domain of a T cell receptor combined with an antigen-recognizing domain [179]. CAR T cells directed against CD19 have been shown to be effective in B cell malignancies [180]. In HL, Wang et al [181] reported a phase I study including 18 patients with relapsed or refractory HL treated with CAR T cells directed against CD30, with seven patients achieving partial remission and six stable disease. Two patients developed grade III or IV toxicity (one elevated liver enzymes and one left ventricular systolic dysfunction). Other possible side effects were nausea/vomiting, urticaria, shortness of breath, psychiatric abnormalities, swollen joints, dizziness and pneumonitis. However, none of the patients developed significant cytokine

release syndrome or tumor lysis, two commonly reported side effects of CAR T therapy.

HDAC inhibitors

Histone deacetylase (HDAC) inihibitors (e. g. panobinostat) induce cell death in HL cell lines [182] and modulate cytokine levels (e. g. TARC) and PD-1 on T cells [183]. Clinically, it has not been as effective as brentuximab vedotin or the PD-1 antibodies, but may have a role in combination therapies [184].

Other possible alternatives besides the classical combination modality treatments are mTOR inhibitors (e g sirolimus), other checkpoint inhibitors (e g LAG-3), anti-PDL-1 antibodies and substances interacting with the deregulated pathways in HL [185, 186].

Aims

Overall aim

The overall aim was to investigate pediatric HL in a Swedish/Nordic cohort with emphasis on disease presentation, treatment outcome, tumor specific characteristics and late effects.

Specific aims

Paper I

To evaluate a cohort of pediatric HL in Sweden over a 25 year time period in terms of disease presentation, changes in treatment and outcome to get an overview of how treatment and care taking of our HL patients has been carried out.

Paper II

To investigate the role of the microenvironment in pediatric HL with special emphasis on EBV-status, mast cells, eosinophils and macrophages.

Paper III

To compare disease presentation and outcome in children, adolescents and young adults (<25 years) and patients treated with pediatric versus adult treatment regimens in a population-based Swedish-Danish cohort.

Paper IV

To compare late effects of treatment in children, adolescents and young adult patients treated with pediatric versus adult treatment regimens, between Sweden and Denmark and between relapsing and non-relapsing patients.

Materials and methods

Study populations

Paper I: The Swedish Childhood Cancer Register was used to identify all patients 0-17 years of age diagnosed with HL in Sweden between 1985 and 2009, in total 335 patients, of which one was excluded due to a non-confirmed HL diagnosis. The study was based on data reported to the register. Additional data on the deceased patients was retrieved from medical records.

Paper II: All patients registered in The Swedish Childhood Cancer Register with a diagnosis of HL between 1983-2008 in Uppsala, Stockholm and Umeå (142 patients) with retrievable tumor material were included in the study, in total 98 patients. Data from the register were complemented with data from medical records.

Paper III: All individuals diagnosed with cHL before the age of 25 in the period 1990-2010 in Denmark and 1992-2009 in Sweden identified in the nationwide Danish and Swedish Cancer registers, were included. Clinical information was available in 1072 individuals with cHL and was retrieved from the Danish and Swedish Childhood Cancer Registers, the Danish Hematology Database, the National Database of HL (Sweden), the Swedish Lymphoma Registry, and from medical records.

Paper IV: The database of patients with available clinical information in paper III was linked to the national population and cause of death registers [187, 188] to ascertain vital status; to national hospital registers [189, 190] to ascertain information on hospital care following HL treatment; and to national cancer registers [191, 192] to ascertain secondary cancers among the HL patients, in total 1045 patients.

Methods

Statistics

Pearsons' Chi^2 test or Fisher's exact test when applicable (dichotomous variables), Mann-Whitney U test (non-parametric comparisons of categorical and continuous variables) and Mantel-Haenszel trend test (comparison of more than two groups) were used to compare groups. Student's T-test was used to compare means between groups. The Kaplan Meier method was used for survival estimates with log rank as significance test when comparing survival in different groups. OS, DFS and EFS were used as survival estimates. Cox regression was used to test the effect of different covariates on OS and EFS. Hazard ratios (HR) with 95 % confidence intervals (CI) were used to compare groups (paper III and IV), both crude and adjusted for different variables. CIs for survival were calculated in the SAS proc lifetest using the default complementary log-log transformation of survival estimates. In study IV the patient groups were defined by baseline characteristics and first line treatment modalities together with the time dependent status as relapsed. The patients first contributed with outcome data in the relapse-free group and then, at first relapse, moved to the relapsed group. The significance level was set to $p<0.05$.

Immunohistochemistry and EBV detection (study II)

We used paraffin-embedded tumor material sectioned in 3-5 μm sections to stain for markers for EBV and the different cells of interest. For EBV, LMP1 staining was used in all cases (anti LMP1, Dako M0897, dilution 1:50, pre-treatment PT Link Envision Flex Target Retrieval Solution High pH, K8000). Those negative for LMP1were complemented with EBER in situ (in situ hybridization for EBV encoded small RNAs). EBV-serology was available in 80 of the cases, analyzed as a routine at time of diagnosis. For eosinophils we used haematoxylin-eosin, for mast cells a monoclonal antibody recognizing tryptase (G3 Chemicon International, Temecula, CA, USA, pre-treatment Proteinase K, S3020, Dako, Glostrup, Danmark) and for macrophages a monoclonal antibody against CD68 (PG-M1 M 0896 Dako, dilution 1:200, pre-treatment as for LMP1).

Quantitative analysis of cell distribution (study II)

Eosinophils and mast cells were counted in ten randomly selected high power fields (HPF) in 400x and 200x magnification respectively. The mast cells were analyzed at a lower magnification to cover a larger amount of the tumor. A lattice square net was used and the absolute numbers of positive cells within the net area were counted. For macrophages, three different regions of the tumor were counted in 400x magnification and the percentage of CD68 positivity in the cytoplasm was scored.

Eosinophils, mast cells and macrophages were analyzed as continuous or categorical variables. For eosinophils the cut off points for categorization were set at 0-9, 10-199 and ≥200 respectively, based on an earlier finding of poorer prognosis in adults in cases with ≥200 eosinophils in tissue [33]. The categorization of mast cells was based on the distribution of mast cells in the material and analyzed as 0-23 vs ≥24 mast cells per 10 HPF and 0-61 vs ≥62 (median and upper quartile as cut off points respectively). Macrophages were counted as a quote of CD68-positive cells relative to overall cellularity and scored as <5 %, 5-24 %, 25-49 % and more than 50 % to allow comparison with results from other groups.

Ethical considerations

The Regional Ethical Review Board in Uppsala, Sweden (all) and the Danish research ethics committee system (papers III and IV) have approved the studies.

Results

For a detailed presentation of the results, see each respective paper.

Incidence of pediatric HL in Sweden

In **paper I,** 334 patients were enrolled. The incidence in the age group 0-14 years was 0.5/100 000 and 0.7/100 000 in the 0-17 year-old group. We could conclude that there was no change in incidence trend during the period studied (*Figure 13.*), although there is a slight variation from year to year. In Sweden approximately 10-15 children and adolescents are diagnosed with HL each year.

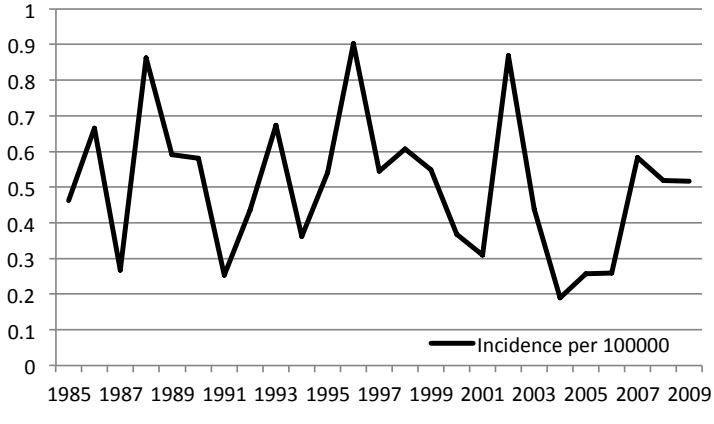

Figure 13. Incidence per 100 000 children, 0–14 years 1985–2009, all subgroups, in Sweden (from paper I [120] with permission).

Clinical presentation

Thirteen per cent of the patients presented with NLPHL (n=42) and 87 % (292) with cHL (**paper I**). The distribution of cHL was: NS 68 % of the total group (n=227), MC 15 % (n=50), LD 1 % (n=4) and no case of LR. Male sex predominated in all subgroups besides NS, where there was a slight

female preponderance (52 %). The same pattern was seen over the age groups, except for the oldest age group 15-17 years where 55 % were girls. MC was more common in the younger age and NS in the older age groups.

The differences between cHL and NLPHL is described further in **paper II** where we could conclude that none of the NLPHL tumors expressed EBV in the tumor cells and that they had less infiltration of eosinophils, mast cells and macrophages around the tumor cells. In addition, the laboratory parameters were less affected.

Adolescents and young adults shared similar clinical characteristics at time of diagnosis, while children <10 years presented with less advanced stage, lower frequency of B-symptoms and extra nodal disease (**paper III**).

Differences in treatment

During the period studied in **paper I** the treatment of children and adolescents has changed, following the different protocols. The most prominent difference was that among the patients treated according to Euro Net PHL-C1, significantly fewer patients received radiotherapy compared to patients treated according to other protocols and those treated with radiotherapy received lower doses.

In **paper III**, when we compared treatment and outcome in Sweden and Denmark we found that Denmark had treated much less with radiotherapy in primary treatment in the pediatric patients (36 % vs. 71 %, $p<0.0001$) and this corresponded to a higher frequency of relapse in this group.

EBV and the microenvironment (paper II)

cHL

The results of the proportion of EBV positive tumors were congruent with earlier published results with MC more likely to be EBV positive than NS (77 % vs 16 % $p<0.001$) and lower mean age among EBV positive cases (EBV positive 10 years, EBV negative 14 years, $p=0.01$). There was a trend for male patients to more likely present with an EBV positive tumor ($p=0.06$).

In the microenvironment analyses we could not detect any significant differences in the number eosinophils among the parameters studied (sex, age (under or over 10 years of age), stage, B-symptoms, EBV status and laboratory parameters), although there was a trend towards higher eosinophil count in more advanced stages (stage III-IV, mean 137, vs I-II, mean 70, $p=0.08$). There were no detectable differences in OS or DFS with regard to presence of high eosinophil count.

Higher mast cell infiltration was seen in advanced stages (stage III-IV, mean n=81, vs I-II, mean n=28, $p<0.001$) and in patients presenting with B-symptoms (mean n=63 vs. n=31, p=0.01). Laboratory parameters in patients with ≥62 mast cells per 10 HPF were affected, with lower hemoglobin and albumin levels and elevated ESR. In cases with mast cell counts over median (≥24 per 10 HPF), ESR and CRP were elevated ($p<0.001$ and p=0.02 respectively), and there was an increase in leucocyte and neutrophil count with borderline significance (p=0.05). There were no detectable differences in OS or DFS with regard to mast cell count, although the number of relapses in cases with <24 mast cells per 10 HPF were lower (4/43) than in those with ≥24 per 10 HPF (8/44). Neither was there any difference observed with respect to sex, age or EBV status.

Most of the cases presented with a macrophage count of 5-24 % (71 cases, 82 %), ten cases (12 %) with <5 %, six cases (7 %) with 25-49 %, no cases with >50 %. More advanced stages had higher macrophage count (stage III-IV, mean 16 %, vs. I-II, mean 10 %, p=0.02). Higher ESR, higher C-reactive protein (CRP) and higher neutrophil counts were seen in cases with > 25 % macrophage count. OS and DFS did not differ between the groups with regard to macrophage count and there were no difference was observed with respect to sex, age or EBV status.

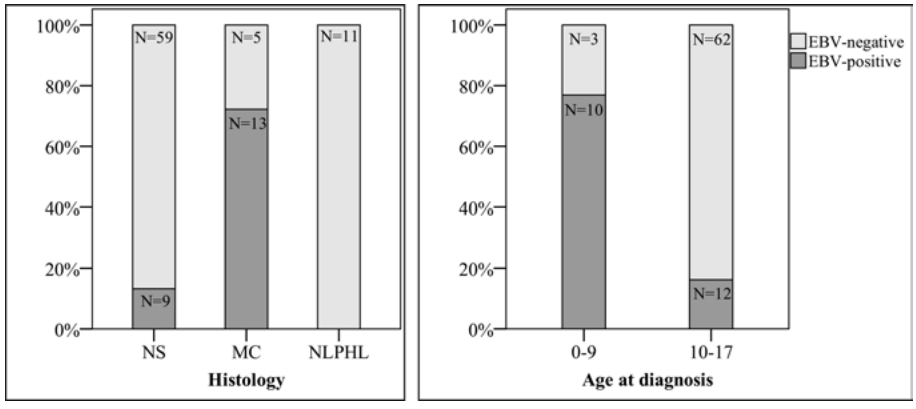

Figure 14. Proportion of EBV-positivity in different sub- and age groups (from paper I [120] with permission).

NLPHL

All NLPHL cases were EBV negative in the tumors. When the cell count results were compared to cHL, NLPHL cases had lower levels of infiltrating mast cells, eosinophils and macrophages. Laboratory parameters in NLPHL cases were less affected, with higher hemoglobin and albumin levels, lower ESR, higher thrombocyte counts and lower leucocyte counts (lower neutrophil but higher lymphocyte count) compared to cHL.

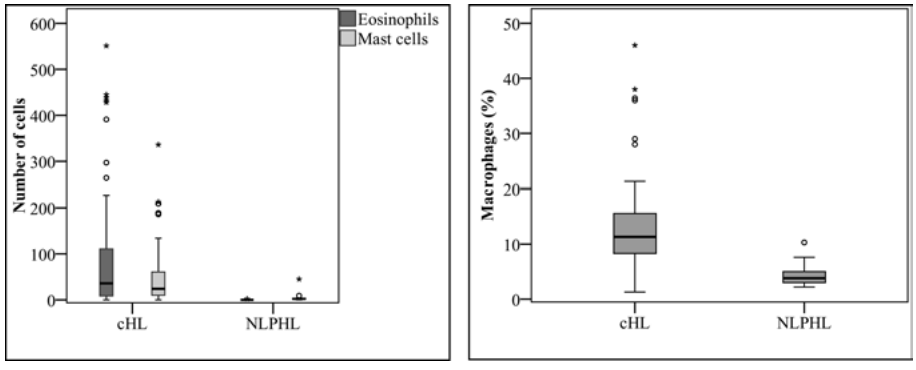

Figure 15. Differences in cell count between NLPHL and cHL (from paper I [120] with permission).

Survival, relapse and causes of death

The 5-, 10- and 20-year OS in Sweden (all cHL patients in **paper I**) was 96±1 %, 95±1 % and 90±3 %, respectively, with a mean follow-up time of 11.4 (0–25.5) years. OS did not differ significantly between boys and girls, different age groups, those with or without B-symptoms or different groups of treatment. Neither were there any survival differences over time when comparing patients treated before and after 1996 (which was when Sweden started to treat according to the different protocols of the GPOH group and the European Euro-Net Paediatric Hodgkin's Lymphoma Group.

There were no significant differences in EFS when comparing the three therapy groups. In therapy group 2 stage IIB had significantly lower EFS compared with the other stages in the same group (stage IIAE, IIIA) (p=0.028). This pattern was seen both in patients treated before 1996 and after, although it was statistically significant only for the whole group and for the group treated after 1996. With cox regression analysis, including the covariates: year of diagnosis, age, sex, stage, B-symptoms and group of treatment (before vs. after 1996), patients with B-symptoms had marginally significant lower EFS (p=0.05).

Relapse occurred in 29 cHL patients, approximately 10 %. The mean and median time from diagnosis to relapse was 1.6 and 1.0 years respectively (0.4-6.5 years).

Eighteen of the 292 patients died, nine from HL and/or other lymphoma. The 5- and 10-year OS after relapse of cHL was 81±8 % and 75±10 % respectively. All NLPHL patients, five of which had encountered a relapse, were alive.

Among the patients treated in therapy group 2, the stage IIB patients accounted for the majority of the relapses, which might indicate that those tumors are more aggressive and might need more treatment.

Table 3 *Causes of death in the Swedish HL population during 25 years (from paper I with permission).*

	NLPHL	**cHL**					**Total**
		NS	MC	LD	LR	Unspec	
All patients	42	227	50	4	-	3	334
Relapse	5	25	3	1	-	0	34
Alive	42	212	48	3	-	3	316
CR-1	37	202	47	3	-	3	300
CR-1 (%)	88	89	94	75	-	100	90
Alive>=CR-2	5	10	1	-	-	-	16
Dead	-	15	2	1	-	-	18
Dead in CR-1 (incl two SMN)	-	6	1	0	-	-	7
Dead from HL (seven relapsed, two progressive disease)	-	7	1	1	-	-	9
Dead due to treatment complications		1	0	0	-	-	1
Dead in CR-2	-	1	0	0	-	-	1

Among those dead in CR-1, two died from a second malignancy (breast cancer and MDS with allogeneic stem cell transplantation, with AML occurring two years after transplantation.) In the remaining four cases the cause of death was not found in the patient charts.

There were no statistically significant differences in EFS or OS comparing children, adolescents and young adults **(paper III)**, with 10-year EFS of 0.93 (95 % CI 0.82-0.97) for children, 0.84 (95 % CI 0.80-0.87) for adolescents and 0.87 (95 % CI 0.84-0.89) for young adults. Pediatric patients in Sweden experienced less events than Danish pediatric patients (10 year EFS 0.82 (95 % CI 0.78-0.86)), Swedish pediatric patients (0.88 (95 % CI 0.84-0.91)), adjusted HR: 1.90 (95% CI (1.03-3.52) (*Figure 16.*). Patients with advanced stage experienced more events, adjusted HR: 1.84 (95 % CI 1.32-2.57) and more deaths, adjusted HR: 2.61 (95 % CI 1.39-4.91) than patients with limited stage.

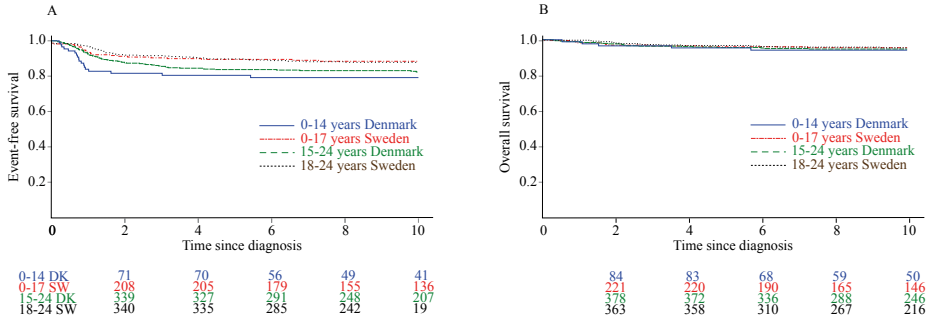

Figure 16. OS and EFS in pediatric and adult patients in Sweden and Denmark.

Late effects of treatment (paper IV)

The number of hospitalization days and outpatient visits was very skewed in the analyzed population, with 10 % of the patients accounting for approximately 80 % of the days in hospital and 50 % of the patients accounting for approximately 90 % of the total number of visits. The relapsed patients accounted for a large proportion of the bed-days in all follow-up periods (except for the first year after diagnosis, where primary treatment was given). Relapsed patients had more and longer hospitalizations.

When comparing incident discharge diagnosed according to ICD10 chapters, relapsed patients were at higher risk than non-relapsed patients of being hospitalized over the whole spectrum of diseases.

The pattern seen in paper III, with a higher frequency of relapses among Danish pediatric patients could not be identified as a higher need of health care use in this group.

A total of 39 secondary cancers were observed among the HL patients during follow-up. Among these, eight (21 %) had previously encountered a relapse, including six cases of cervical cancer, five cases of breast cancer, five cases of myelodysplastic syndrome/acute myeloid leukemia, and 11 cases of skin cancer.

Discussion

The first study (**paper I**) was performed to evaluate the treatment results and outcome among Swedish patients with HL over a long time period, during which different treatment strategies have been used, to get an overview of how treatment and care taking of pediatric HL patients have been carried out in Sweden. Other chemotherapeutic drugs are used in contemporary protocols than in the first years of the study cohort and radiotherapy is given to a lesser extent, the intent being to diminish late effects of treatment without poorer outcome. Hence, one of the main aims of this study was to evaluate the outcome over a long time to detect any eventual deterioration in survival estimates.

We were not able to detect any differences in OS or EFS over time during the period studied. However, in the subgroup analyses for each therapy group there was significantly lower EFS in stage 2B compared to the other stages in the same therapy group. This could be interpreted that B-symptoms are more important for risk of event than is the number of engaged sites. In adult HL patients in Sweden, especially those with bulky disease [193, 194], a similar pattern has been described, which resulted in an up grading of the treatment in those patients. In the register used in our study there are, however, no reliable data on bulky disease. Patients in stage 2BE with extra nodal disease are treated more intensively in therapy group 3. It may be worth considering whether all stage 2B patients should be treated with higher intensity. However, this observation is based on a few events and over a long period of time and has to be interpreted with caution.

There are few events in HL. Hence, the power to detect small changes in survival is limited and, especially when no difference has been detected, the results must be interpreted with caution and equivalence cannot be assumed. Thus, large cohorts are needed to detect changes in OS and EFS, which emphasizes the need for good collaboration worldwide and inclusion of patients in randomized trials when possible.

The study supports the description of NLPHL as a separate entity of HL with very good prognosis. All patients included are alive at follow-up.

There is a good chance of survival even after relapse in Swedish pediatric patients with HL. The treatment results in Sweden are good, comparable to the most recent pediatric trials summarized by Kelly et al. [195].

The microenvironment in HL has been given a lot of attention with its peculiar pattern of a few tumor cells surrounded by a large mass of inflammatory response. Several studies have addressed this field in adults, but only a few studies in children. In **paper II** patients with high mast cell and high macrophage counts were more likely to present with parameters associated with more advanced disease, such as stage and B-symptoms. However, there were no detectable differences in OS or EFS. These results could be interpreted in at least three ways; either the cell count of inflammatory cells is not of importance for outcome, or the cell count is of importance for risk but is in this case taken care of by increased treatment resulting in similar outcome, or the cell count actually does have an impact but our study was underpowered to detect it. The material also spans over a long period of time, with different treatment strategies. The ideal study design would be to study a uniformly treated cohort, but this is difficult in rare diseases and low numbers of affected children, which, once again, emphasizes the importance of good research network and collaboration.

Published studies of differences in microenvironment between NLPHL and cHL are scarce. In this study the two entities clearly differ, both in clinical parameters and in the microenvironment, which further supports the view that NLPHL is a separate entity of HL.

The study in **paper III** was conducted in an attempt to add light to the discussion on whether pediatric or adult treatment regimens should be used in the adolescent/young adult group [196]. There are a few studies including adolescents in adult trials [197, 198], where treatment results have been satisfying. A better EFS and OS in patients <18 years of age treated with pediatric protocols was suggested by Muller et al [199]. The finding that adolescents and young adults shared similar treatment characteristics in our study would support a harmonization of treatment for these patients. We did not find any differences between adult and pediatric treatment regimens, but an unexpected difference in EFS between Swedish and Danish pediatric patients and also a striking difference in the use of radiotherapy, which could possibly lead to a lower burden of late effects.

The study in **paper IV** was inspired by the results in paper III and provides information on hospitalization rates and late effects with background characteristics and outcome, making it possible to give a more detailed picture of the need for hospital in- or outpatient visits than what is generally provided in similar studies. Either relapse information has not been provided [134, 200-203], giving a very general picture of all survivors, or relapse was considered but patients treated since 1999 were not included [204-207], and thus not reflecting contemporary treatment.

Most of the young survivors of HL have low burden of late adverse effects and the relapsing patients are the main drivers of excess need of health care use.

Whether those survivors who accounted for a low percentage of bed-days and health care still encounter a higher risk than the general population cannot be concluded from this study due to its design without an external comparison group. The differences seen in paper III in treatment and EFS among pediatric patients were however not manifested in differences in hospitality rates and late adverse effects.

This study reflects contemporary treatment and has a relatively long follow up. However, some late adverse effects take a long lag time to occur and might not have occurred during the follow up time.

Strengths and limitations

The quality of the population-based registers in Sweden is well known and gives us the possibility to study large cohorts. It also assures the quality of the care and treatment given.

Our clinical registers and the possibility to collect tumor material retrospectively from the different biobanks also allow for studies of tumor biology and etiology.

The pathological re-evaluation of nearly 100 cases in study II revealed a low percentage of misclassifications, reassuring the high quality of pathologists in Sweden.

The Cancer Register (mandatory by law) has somewhat more cases registered than the clinical registers that are non-compulsory and which thus rely on the patients consent and on the clinician to report data. This might lead to some bias in studies based on clinical registers; e.g. false high survival rates due to the fact that patients who die shortly after diagnosis might not be reported.

Register-based studies have certain limitations, e. g. that they cannot provide as much information and stratification as a randomized trial, but rather give an overview of the patterns over time.

Studies on late effects all have the same problem, i. e. the long lag time from the end of treatment until the diagnosis of treatment-related late effects, which makes it difficult to evaluate the contemporary treatment.

Survival studies on HL in pediatric patients in Sweden are challenging. The high survival rates and relatively low risk of relapse combined with low incidence in a small country make in difficult to achieve power in the analyses. Sweden in itself cannot conduct studies on pediatric HL due to this limitation. Thus, collaborations such as NOPHO and European/International networks are of the outmost importance.

Conclusions

- Hodgkin lymphoma incidence in children and adolescents in Sweden has been stable over the past three decades with an absolute number of 0.5-0.7/100 000 individuals.

- Swedish children and adolescents have a survival after HL comparable to the best results in the world and there is a good chance of cure even after relapse.

- The microenvironment in terms of eosinophils, mast cells and macrophages is reflected in the clinical presentation, but is not a strong predictive factor in this Swedish pediatric cohort.

- The clear difference between cHL and NLPHL and the fact that all patients with NLPHL in Sweden during the period studied are alive supports the ongoing efforts to reduce treatment in this specific group of patients.

- The similar clinical presentation of adolescents and young adults with HL would support a harmonization of treatment in these two groups. Both pediatric and adult treatment protocols provide high survival rates and it might not be possible to define the best treatment.

- The different treatment strategies in Denmark and Sweden in terms of usage of radiotherapy was reflected in the outcome with a higher frequency of relapses, but the comparable overall survival confirmed effective relapse treatment.

- Late effects from cancer treatment are complex to study due to the long time it takes to develop e. g. a secondary malignancy. Hospitalization rates are a proxy of treatment burden that is often used in studies to evaluate more contemporary treatment. However, we could conclude that it is of great importance to consider relapse when conducting such studies. Otherwise, the impression might be that all patients are at great risk of late morbidity, when it in fact is just a small proportion of the patients that is carrying a large burden.

Future perspectives

The microenvironment in HL is of great interest and to further explore the interactions around the tumor cells is crucial to be able to solve the enigma of its appearance. In a pediatric cohort it would be interesting to further investigate the role of PD-1, PD-1 ligand and regulatory T cells and their impact on prognosis and clinical presentation. The challenge is to create a large enough sample size to earn power in the survival studies considering the relatively low incidence and excellent prognosis.

In the clinical field, the development of novel targeted drugs is promising. One challenge now is to evaluate their role in the treatment of pediatric patients and whether they should have a role in primary treatment regimens. For such studies it is absolutely necessary to include our patients in large studies, preferably in pediatric international collaborations or, alternatively in collaboration with adult oncologists. At least for the young cohort (adolescents and young adults) it would be reasonable to harmonize the treatment between pediatric and adult clinics.

The excellent quality of our registers in Sweden and the possibility, through the personal identity number, to link the registers to each other and to compare with matched controls, is an interesting source of valuable information that would be motivating to use in future studies e g. future parenthood, sick leave, employment, use of pharmaceutical drugs.

The new establishment of a national biobank for pediatric malignancies will hopefully facilitate biological studies.

The NOPHO organization, where we use the same treatment protocols and have collaborative groups with all countries represented, is a valuable platform for future studies on pediatric HL.

Sammanfattning på svenska

Hodgkins lymfom (HL) hos barn, ungdomar och unga vuxna är en sjukdom som med dagens behandling har god prognos. Epstein-Barr virus (EBV) uttrycks i en del av tumörerna och är där en orsaksfaktor till uppkomsten. Vad som gör att HL uppstår hos dem där tumören inte uttrycker EBV är ännu okänt. Tumörerna består av ett fåtal tumörceller omgivna av många inflammatoriska celler, där de senaste årens forskning visat att dessa celler har betydelse för hur tumören utvecklas och hur aggressiv sjukdomen är. HL drabbar personer i alla åldrar men har två ”pucklar” där insjuknandefrekvensen är högre. Den första ”puckeln” ligger hos tonåringar och unga vuxna och den andra puckeln hos vuxna över 50 år. Behandlingen är ofta en kombination av cytostatika och strålbehandling och i vissa fall också antikroppar mot tumörcellerna. Upplägget av behandlingen förändras kontinuerligt, där syftet idag är att bibehålla den goda överlevnaden och minimera risken för sena komplikationer av behandlingen. Med dagens behandlingar riskerar en ansenlig andel av patienterna sena biverkningar så som sekundära maligniteter, hjärt/kärlsjukdom, lungpåverkan, endokrinologiska störningar och infertilitet. Behandling av sjukdomen sker på barn- eller vuxenklinik beroende på ålder vid insjuknandet, där övergången till vuxensjukvården i Sverige sker vid 18 års ålder. Behandlingsprotokollen på de olika enheterna skiljer sig åt både med avseende på typ av cytostatika och kriterier för strålbehandling.
I min avhandling har jag fokuserat på Hodgkins lymfom hos barn, ungdomar och unga vuxna ur flera olika perspektiv, som beskrivs under respektive delarbete.

Delarbete I

Här har vi tittat på alla patienter <18 år som behandlats för Hodgkins lymfom i Sverige 1985-2009 och hur det har gått för dem. Överlevnaden var god efter avslutad behandling (ungefär 95 % efter 10 år). Cirka tio procent fick återfall, men även efter återfall var överlevnaden god. Sex procent av patienterna hade gått bort vid uppföljningen (18 stycken, varav nio dog till följd av sin sjukdom, en under pågående behandling på grund av komplikationer, två i cancersjukdom till följd av lymfombehandlingen (bröstcancer, leukemi), två hade underliggande, komplicerande sjukdom (immunbrist, hemolytisk anemi). För fyra patienter var dödsorsaken okänd).

Sammanfattningsvis kunde vi konstatera att överlevnaden efter insjuknande i Hodgkins lymfom som barn eller ungdom är god, i nivå med de bästa rapporterade resultaten i världen och att chansen till bot är mycket god även efter recidiv.

Delarbete II

I denna studie har vi fokuserat på mikromiljön i tumören och tittat på andelen EBV-positiva tumörer och antalet inflammatoriska celler (eosinofiler, mastceller och makrofager) i tumörerna och kopplat resultaten till information från journalanteckningar och laboratorieprovsvar. Tumörmaterial från diagnostillfället samlades in och färgades för olika markörer för EBV och de olika celltyperna. Vi jämförde sen olika grupper (kön, ålder, utbredning (stadium), EBV positiv/negativ) Vi fann att yngre barn hade större sannolikhet för att uttrycka EBV i tumören, vilket också hängde samman med subtypen mixed cellularity. För celltypen eosinofiler kunde vi inte hitta några skillnader i cellantal mellan olika grupper och ingen skillnad i överlevnad eller risk för återfall relaterat till antalet eosinofiler i tumören. Hos dem med mer avancerad sjukdom (högre stadium) sågs högre antal mastceller och makrofager och mer påverkade laboratorievärden. Vi såg ingen skillnad i överlevnad eller risk för återfall. Så kallad klassisk HL skilde sig tydligt från subgruppen NLPHL. Ingen av NLPHL tumörerna uttryckte EBV och hade färre antal eosinofiler, mastceller och makrofager i tumörerna. Patienter med NLPHL hade också lägre grad av påverkan i laboratorieprover.

Delarbete III

Beroende på ålder vid diagnos behandlas barn, ungdomar och unga vuxna med Hodgkins lymfom antingen vid en barn- eller vuxenklinik. Behandlingsprotokollen skiljer sig åt både med avseende på de cytostatika som används och vad som avgör om strålbehandling ska ges eller inte. Detta medför att personer med identisk sjukdomspresentation, men på olika sidor om åldersgränsen (i Sverige 18 år), får olika behandling.

Vi har, i ett samarbetsprojekt med Danmark, jämfört sjukdomspresentation hos barn (<10 år), ungdomar (10-17 år) och unga vuxna (<25 år) och tittat på given behandling och prognos. Ungdomar och unga vuxna hade liknande sjukdomspresentation vilket skulle stödja en harmonisering av behandlingen i dessa grupper. Barn < 10 år hade lägre sjukdomsutbredning och större andel pojkar.

När vi jämförde överlevnad kunde vi inte hitta några skillnader mellan olika åldersgrupper men en tendens till lägre risk för återfall/död hos de yngsta barnen. Behandling vid barn- och vuxenklinik skilde sig inte heller åt

gällande överlevnad. När vi jämförde länderna mot varandra kunde vi dock konstatera att patienter behandlade på barnklinik i Danmark hade högre risk för återfall. Det var dock ingen skillnad i överlevnad, talande för att behandlingen vid återfall var effektiv. Den mest uppenbara skillnaden i behandling mellan de olika länderna var en högre grad av strålbehandling i Sverige. Det här var en skillnad vi inte väntat oss då Norden i stor utsträckning följer samma protokoll och inkluderar i studier när det är möjligt. Skillnaden i behandling understryker vikten av att inkludera patienter i studier när det är möjligt.

Delarbete IV

Sena biverkningar av behandlingen mot Hodgkins lymfom har studerats extensivt och ett stort antal studier beskriver ökad frekvens av cancer, hjärt-kärlsjukdom, lungpåverkan, endokrinologiska störningar, nedsatt förmåga till fortplantning och ett ökat behov av sjukvårdskontakter och sjukhusvård. Huruvida detta gäller alla patienter eller endast vissa är ofullständigt studerat då man i flertal studier inte tagit hänsyn till återfall i sjukdom vid analyserna.

I delarbete IV har vi utgått från databasen i delarbete III och tittat på hur behovet av sjukvårdskontakt är fördelat. Sammanfattningsvis kunde vi konstatera att ett litet antal patienter stod för en stor del av sjukvårdsbehovet. En stor grupp hade inget behov av sjukhusvård. Dessa fynd är viktiga av flera anledningar. Det är viktig kunskap att många faktiskt inte behöver mycket sjukvård efter att ha blivit botade, både för patienten, men även för hur sjukvården ska planera sin uppföljning. Den lilla grupp som behöver mycket sjukvård behöver vi fokusera mer på. Att finna faktorer som bättre kan förutsäga vilka patienter som riskerar att få återfall och vilka som riskerar att drabbas av en tung sjukdomsbörda är här en viktig del.

Acknowledgements

This thesis was financially supported by the Swedish Childhood Cancer Foundation, the Mary Beve Foundation, the Lions Cancer Research Fund, the Selanders Foundation and the Gillbergska Foundation.

I have been supported by a lot of people during the work with this thesis. Among those I would like to thank in particular are:

Gustaf Ljungman, colleague and my main supervisor, both in research and in the clinical field. Working with you is like paddling a canoe with you sitting in the back of the boat. I need to paddle hard to keep up with your pace, but when I´m not strong enough, you´re always there to help keeping the pace and the direction right.
Gunilla Enblad, co-supervisor. Your enthusiasm for research in the field of Hodgkin lymphoma is never fading and it is severely contagious. You were the one who got me in to this from the beginning.
Rose-Marie Amini, co-supervisor. You actually make me feel that pathology is the most interesting thing on earth… "If you have a bad day, you can always take a slide of Hodgkin lymphoma and put it under the microscope. It will make you feel better, because it is so beautiful." That is a true pathologist and researcher.
Daniel Molin, co-supervisor. Sending an e-mail to you is like throwing a snowball at a goalkeeper in hand ball. It comes back and "hits" you with an answer before you have even pressed send.
Ingrid Glimelius, my friend and co-author. You are a "fast thinker", but your tongue is even faster. We all love "the sweet little frogs" jumping out of your mouth. Working with you is never hard.
All **co-authors,** mentioning in particular "the Danish team" **Lisa and Henrik Hjalgrim,** for your admirable skills in both the clinical field and in research, and for your hospitality and **Klaus Rostgaard** for your statistical excellence- "You are like PCR with numbers. You get a small amount of numbers and amplify it into thousands in a second!"

Britt-Marie Frost, colleague and the head of the pediatric hematology and oncology unit in Uppsala, for providing an excellent atmosphere for research and clinical work in our department, for using your warm personality when leading our team and for sharing your experiences, professional and private.

My colleagues in pediatric hematology and oncology in Uppsala for providing an open and joyful atmosphere at work and for your professional excellence: **Johan Arvidson**, for sharing your deep knowledge in pediatric oncology as well as your knowledge of all operas around the world; **Per Frisk** for guiding me in the mysterious field of hematology with your subtle sense of humor; **Britt-Marie Frost** (mentioned above), **Clary Georgantzi**; for bringing some glamour to our group and for working Christmas Eves (!); **Britt Gustafsson** for valuable input in the care of our transplanted patients and inspiring stories about working abroad; **Natalja Jackmann**, for your enthusiasm in all fields and for your admirable language skills; **Gustaf Ljungman** (mentioned above); **Josefin Palle**, for sharing experiences in combining work, motherhood and life; **Anders Öberg**, my wingman in taking my first steps into pediatric oncology, for kindly asking easy questions, thus making me feel like an expert in computer based tools, such as "End Note"; **Åke Jakobson** and **Birgitta Lannering**, for keeping up the working spirit and for valuable contribution of your deep knowledge in the pediatric oncology field.

Tove Kamsvåg Magnusson and **Jenny Thorsell Cederberg** for excellent company (including chocolate!) and nice chats "på forsket" throughout the process with this book.
All **research nurses** in all the pediatric oncology units in Sweden, and in particular the "Uppsala gang" for your eminent reporting to our registers, making it possible to do population-based studies.
All **the staff at 95A**, the unit for pediatric hematology and oncology in Uppsala. I am proud of the teamwork in our unit and of all the knowledge in different fields of medical care you provide when taking care of our patients. Everyday you make a difference!
The VSTB-team (Vårdplaneringsgruppen för Solida Tumörer hos Barn) for keeping up with the latest therapy recommendations for Swedish children suffering from a solid tumor.
All other colleagues at **Akademiska Barnsjukhuset** for taking care of the sick children in Uppsala with engagement and expertize.

All my dear, dear friends, sorry that I cannot mention all of you by name, or even by "group", for chats, vacations, parties and important "deep talk":
Friends from **"way back**", with whom I grew up together "hemma på gatan", in school, "pen-paling" etc. You know everything about me.
All friends from **medical school**, my private and professional network. It really thrills me and fills me with joy when we meet and I see our kids play and to see them grow up as reincarnations of you all.
Bokcirkeln, for keeping up with nice chats and food with or without babies and big bellies. Sometimes we even read the book, which is not the most important thing.

The old "Snerikekören" – gang for not giving up on me attending the yearly reunion and for the "möhippa utan brud" occasions.
All my other friends that do not fit in the above defined groups, you are of course also in my mind.

Eva Oldinger, mother in-law, for long discussions about everything in life, for being a wonderful grandmother and helping out whenever we need it; **Hans Englund**, father in-law, for sharing your enthusiasm and deep knowledge about the archipelago and its animal life with us and our children and for all the exciting stories about "farbror Albert", told early in the morning; **Ulrika Rasmusen** "step mother in-law" for nice discussions while kayaking and for being a great "summer grandma"; **Ninni Englund** with family and **Pia Englund** with family, my sisters-in-law, always a step ahead in life and parenthood, showing us what joys and challenges we are approaching.
"Djungeltelegrafen", relatives on my mother's side. All news spreads like wildfire. Thank you for fruitful discussions, the "bilakuten/barnakuten" exchange, skiing together traditions, for being there in good and in bad times.
Rolf and Monika Edström, my parents. Without your endless love and support in all fields always this book would never have been written. Thank you for everything you do for us and for our children.
Lena Edström, my beautiful and talented sister. I love having you and your family David, Tuva and Malva just a few steps away.
Olle Englund, my husband and partner in life. Living with you is a wonderful journey and, as you have promised me, never boring ;).
Rebecka, Valter and **Jonatan**, our three lights in life. You are the future.

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