CLINICAL STUDIES OF HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

HELENA TROTTESTAM

Stockholm 2011
To my father

Till Pappa Olle, som kombinerade bildning och forskarskicklighet med ett fullständigt ointresse för att göra akademisk karriär. Jag önskar att jag kunde ha fått din åsikt om den här boken; jag tillägnar den dig.

"Utan tvivel är man inte klok.”
/Tage Danielsson
ABSTRACT

Background and aims: The term hemophagocytic lymphohistiocytosis (HLH) comprises two main disease entities: the primary, familial form (FHL) and an acquired, secondary form (sHLH). FHL is autosomal recessive in inheritance, typically affects very young children and is almost invariably fatal unless treated. Secondary HLH typically occurs in older children and adults. However, sHLH may also affect infants and FHL may affect adults. In the absence of reliable functional cell studies, a genetic diagnosis or a family history of HLH, differentiation between the two at onset is virtually impossible. Other inherited syndromes in which HLH can develop are X-linked lymphoproliferative disease (XLP), Chédiak-Higashi syndrome (CHS), and Griscelli syndrome type II (GS2). HLH signs and symptoms are related to hyperinflammation after a triggering infection, and include persisting fever, hepatosplenomegaly and non-malignant infiltration in many organs, including bone marrow, liver, spleen and central nervous system (CNS) of activated T lymphocytes and macrophages, the latter involved in hemophagocytosis. Neurological symptoms can be present already at onset. Laboratory investigations typically reveal cytopenia, elevated values of liver enzymes, ferritin, cytokines and triglycerides, and a coagulopathy. In FHL, natural killer cells are normal in number but show reduced or absent function.

The aims of this thesis include description of disease characteristics at onset as well as presentation of treatment results prior to and after hematopoietic stem cell transplant (HSCT) in children with HLH (Paper I), and in the other inherited HLH-related diseases (Paper III). Furthermore, the aims include description of the frequency and character of acute CNS disease and of CNS sequelae (Paper II), and to evaluate risk factors for adverse outcome (Papers II, IV and V).

Methods: The studies were conducted on data from patients recruited from the databases of the international treatment study protocols HLH-94 (paper I, n=249; paper II, n=193) and HLH-2004 (paper V, n=297). In paper III patients were recruited from both protocols. In paper IV, the data were collected as part of a European collaborative effort.

Results: At a median follow-up of 6 years, overall 5-year probability of survival in the HLH-94 treatment study was 55%, and 5-year survival post-transplant was 67%. Altogether, 73% of the patients received transplants or achieved long-term remission without HSCT. There was no significant difference in survival for patients with familial disease. Patients with a presumed secondary disease were older, more often female, and less frequently had CNS disease at onset (paper I). CNS disease at onset was common, (63%) and an important risk factor for both death and neurological late effects. Neurological sequelae were present in 15% upon follow-up (paper II). Seven of nine patients with GS2, XLP or CHS could receive transplants after HLH therapy, and one had long-term remission without HSCT. At last follow-up (mean 6 years), eight of nine were alive (paper III). Risk factors for early pre-transplant death that remained significant in both papers IV and V were hyperbilirubinemia at onset and hyperferritinemia and thrombocytopenia after two weeks.

Conclusion: In conclusion, survival has increased dramatically in patients with HLH with the introduction of the HLH-94 treatment protocol, but a large proportion of patients still succumb to the disease. Novel treatment strategies need to be developed in order to reduce early pre-transplant mortality, but also to reduce transplant-related mortality and morbidity. CNS-HLH is frequent, and a risk-factor for adverse outcome. Thorough evaluation of acute CNS symptoms and signs, as well as close neurological follow-up is important. Patients with HLH-associated syndromes may also benefit from HLH-94 treatment. There are simple laboratory parameters that may help in risk estimation of HLH patients, and these - alone or taken together - may be used to adapt treatment intensity.
The thesis is based on the following publications. The articles will be referred to by their Roman numerals.


**Chemoimmunotherapy for hemophagocytic lymphohistiocytosis: long-term results of the HLH-94 treatment protocol.**

*Blood. September 6, 2011; E-pub ahead of print*


**Frequency and spectrum of central nervous system involvement in 193 children with haemophagocytic lymphohistiocytosis.**

*Br J Haematol. 2008 Feb; 140(3):327-35*


**Treatment of the X-linked lymphoproliferative, Griscelli and Chédiak-Higashi syndromes by HLH directed therapy.**

*Pediatr Blood Cancer. 2009 Feb; 52(2):268-72*


**Risk factors for early death in children with hemophagocytic lymphohistiocytosis.**

*Acta Paediatr. Accepted for publication October 19, 2011*


**Clinical prognostic risk scoring of patients with hemophagocytic lymphohistiocytosis.**

*Manuscript*
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LIST OF ABBREVIATIONS

AML  Acute myeloid leukemia
APC  Antigen-presenting cell
ATG  Anti-thymocyte globulin
CHS  Chédiak-Higashi syndrome
CI   Confidence interval
CMV  Cytomegalovirus
CNS  Central nervous system
CSA  Cyclosporin A
CSF  Cerebrospinal fluid
CT   Computed tomography
CTL  Cytotoxic T lymphocyte
EBV  Epstein-Barr virus
FHL  Familial hemophagocytic lymphohistiocytosis
GS2  Griscelli syndrome type II
GvHD Graft versus host disease
HLH  Hemophagocytic lymphohistiocytosis
HPS2 Hemansky-Pudlak syndrome type II
HR   Hazard ratio
HSCT Hematopoietic stem cell transplant
IAP  Inhibitor of apoptosis-protein
IFN  Interferon
IL   Interleukin
MAHS Malignancy-associated hemophagocytic lymphohistiocytosis
MAS  Macrophage activating syndrome
MHC  Major histocompatibility complex
MRI  Magnetic resonance imaging
MTOC Microtubule-organizing center
MTX  Methotrexate
NK cell Natural killer cell
PRES Posterior reversible encephalopathy syndrome
RIC  Reduced-intensity conditioning
SAP  Signaling lymphocytic activation molecule (SLAM)-associated protein
sHLH Secondary hemophagocytic lymphohistiocytosis
SoJIA Systemic onset juvenile idiopathic arthritis
TCR  T cell receptor
TNF-α Tumor necrosis factor-alpha
VAHS Virus-associated hemophagocytic lymphohistiocytosis
VP-16 Etoposide
XIAP X-linked inhibitor of apoptosis-protein
XLP  X-linked lymphoproliferative syndrome
INTRODUCTION
Foreword

"Medea took her unsheathed knife and cut the old man’s throat letting all of his blood out of him. She filled his ancient veins with a rich elixir. Received through his lips and wound, his beard and hair no longer white with age, turned quickly to their natural vigour, dark and lustrous; his wasted form renewed, appeared in all the vigour of bright youth".
Ovid may have been the first author relating to a blood transfusion when he wrote his seventh book of the Metamorphoses in 8 AD, describing how Medea rejuvenated Jason's father Aeson. Today, blood transfusions and hematopoietic stem cell transplantations play important roles in the treatment and cure for hematopoietic and malignant diseases.

This thesis is a study of hemophagocytic lymphohistiocytosis (HLH), a rare disease affecting the homeostasis of the immune system; a disease that requires blood transfusions and often hematopoietic stem cell transplantation for cure.

The first description of the disease was probably in 1952, when Farquhar and Claireaux reported on two siblings with a condition with onset at nine weeks of age, and with a rapidly fatal course. In the following decades similar cases were described, and the picture of a hereditary, invariably fatal disease affecting infants in the first year of life emerged. The symptoms were those of an unbridled inflammation, with fever, enlarged liver and spleen, cytopenia, and often pathological findings in the cerebrospinal fluid (CSF) as a result. Pathology examination showed infiltration in various organs by macrophages and lymphocytes, typically involved in hemophagocytosis. In the mid-eighties, more efficient treatment regimens for children with HLH were reported, and an extensive international treatment collaboration was launched in the nineties. Today many, but far from all, patients can be permanently cured of the disease.

HLH treatment trials provide examples of fruitful international collaboration, necessary for the understanding of a rare disease. Furthermore, HLH proves the importance of a lively interchange of knowledge between clinical and pre-clinical research, as the syndrome was first described by clinicians, leading the way for discoveries of molecular pathogenetic mechanisms involved in the disease, subsequently resulting in new clinical tools for diagnosis and treatment.

What were magical arts in the days of Ovid are now medical realities. Most children with HLH can be cured today, but a large proportion still succumbs to the disease. My hope is that this thesis may provide a pixel to the picture that will show us how to improve diagnostic and therapeutic approaches in HLH further.

ENEBYBERG, October 2011

Helena Trottestam
General background

Figure 1. Schematic overview of the hematopoietic system.
**The immune system**

*Immunis* is Latin, and means exempted or protected. The cells that constitute the immune system serve as the body’s defense against foreign intrusion, but also as a protection against damaged elements of the body itself. This is a sophisticated and well-orchestrated system, characterized by specificity, selectivity, adaptivity and memory, and in which small shifts in function can lead to devastating consequences.

Our first-line defense is the innate immune system, which is present from birth. It consists of natural barriers, such as the epithelial layer of the skin, of components of the complement system, anti-bacterial peptides, cytokines and of immune cells such as monocytes, macrophages, mast cells, dendritic cells, granulocytes and natural killer (NK) cells. These cells can act immediately when an infected cell is encountered. As part of a more complex immune response, the immune system adapts over time to recognize specific pathogens more efficiently. This is accomplished through the adaptive immune system, consisting of B cells (humoral immunity) and T cells (cellular immunity). The adaptive immune response is specific for a particular pathogen, but takes longer time to be fully activated, often several days. However, it is also characterized by memory – some of the offspring of the T and B cells remain as memory cells in the blood, to warrant a quicker and more efficient response upon subsequent encounters with the same pathogen.2,3

All blood cells arise from hematopoietic progenitor cells in the bone marrow (*Figure 1*). Primitive hematopoietic stem cells can give rise to all kinds of blood cell lineages as a result of cytokines, transcription factors, cell-to-cell-interaction or through other influence of the microenvironment. In addition to having the capacity to develop unipotent stem cells, they can also renew themselves. However, only a minority is in replication, as opposed to unipotent stem cells that are characterized by constant activity in the cell cycle. The unipotent stem cells, designated to form a certain type of cell, lack the capacity of self-renewal. They proliferate and differentiate as a result of specific colony stimulating factors. Through successive steps the cells divide and mature in the bone marrow. Once mature cells in the blood, the capacity to divide further is lost.4

Myeloid progenitor cells give rise to megakaryocytes, the precursors of thrombocytes, to proerythroblasts, that mature through a number of steps into reticulocytes and eventually into red blood cells, to myeloblasts, that undergo maturation to form mature granulocytes (basophil, neutrophil, or eosinophil granulocytes) and to monocytes. Monocytes that migrate into tissue acquire new traits, and in turn develop into dendritic cells or macrophages. All three cell types of the monocyte/macrophage/dendritic cell lineages are capable of engulfment of target cells, serve as antigen-presenting cells (APC) and are involved in production of cytokines.5

Macrophages are the voracious omnivores of the immune system, that recognize target cells through opsonisation (recognition of intermediary proteins such as antibodies or complement factors), or through direct binding to the target via receptors. After recognition, target cells are engulfed and broken down into fragments; fragments that can subsequently be presented on their cell surface.5 Dendritic cells have tentacles, dendrites, and are also active in phagocytosis, but less effectively than the macrophages.6 However, they excel in antigen-presenting. In contrast to macrophages, they can migrate far from the site of infection to the lymph nodes, where they can activate T lymphocytes.7 However, they are also important for inactivation of T cells and in down-regulation of immune responses in order to maintain immunological tolerance.5 The term
A histiocyte refers to cells of either macrophage or dendritic cell lineage. A subtype of dendritic cell is the Langerhans cell, predominantly found in the epidermal skin layers.

Lymphoid progenitor cells give rise to the lymphocytes that constitute our adaptive immune system. The lymphocytes are T cells, NK cells and B cells.

**T cells**

T cells, or T lymphocytes, are important for activation of other immune cells and for the defense against intracellular impostors, such as viruses. T lymphocytes recognize major histocompatibility complex (MHC) molecules on other cells. The MHCs are highly polymorphic molecules located on the cell surface that display fragments of proteins made in the cell (MHC type I) or caught by the cell by endo- or phagocytosis (MHC type II). MHC type I is present on all cell types, whereas MHC type II is present only on B cells and APC. When the T progenitor cells mature, this is done in several steps: an immature cell is transported from the bone marrow to the thymus where it turns double-positive for the surface antigens CD4 and CD8 and undergoes an extensive proliferation and formation of a T cell receptor, TCR. When a thymocyte by coincidence produces a TCR that fits a MHC-antigen in its vicinity it is positively selected, differentiated into CD4+ or CD8+ T cell, depending on which class MHC molecule it has encountered, and enters the blood circulation. Should it instead happen to produce a self-reacting TCR it is negatively selected, and undergoes cell death. An equal fate awaits the cell who fails to produce a TCR with affinity for an antigen (death by neglect).

The TCR is thus the tentacle with which the mature T cell investigates the cells it meets during its course of lifetime. CD8+ T cells, so called cytotoxic T lymphocytes (CTL), are activated by encounter of their specific combination of MHC-class I molecule and antigen, and CD4+ T cells, so called T helper cells, by their specific combination of MHC-class II molecule and antigen. Cytokines required for T cell activation are secreted from macrophages and APC in the vicinity, and in addition, cell surface signals that synergistically infer with the TCR-MHC-interaction help activation. Once activated, the T cell grows in size and starts production of the cytokine interleukin (IL)-2 and expression of the IL-2-receptor on its own cell surface. After this autocrine loop of stimulation, clonal expansion takes place and an army of T cells are ready to eliminate the identified enemy cells carrying foreign MHC.

In this manner mature T cells learn to discriminate between “self” and “non-self”, i.e., between their host and foreign or damaged elements. This is crucial to avoid a civil war within the body, and we call this self tolerance.

**Natural killer cells**

NK cells are important in tumor surveillance and for control of viral infections. Traditionally, NK cells have been considered part of the innate immune system, but increasing evidence suggests that they share adaptive features with the other cells derived from the lymphoid cell lineage. Alike T cells, NK cells are “educated” and selected during their development, and also often express the CD8-surface antigen. In contrast to CTL, NK cells that do not engage with a MHC class I-molecule during development do not undergo death by neglect, but are released into the periphery and can be “re-educated” at a later time-point. NK cells recognize MHC class I antigen (and lack thereof: “missing self” markers). However, they lack the TCR, and are
activated by antibody-binding with their Fc-receptor molecule, as well as ligation with various other regulatory receptors on the cell surface. Furthermore, NK cells may undergo clonal expansion during infection and they generate long-lived memory cells.

**B cells**

B cells, or B lymphocytes, are the other important players of the adaptive immune response. They form and mature in the bone marrow and, much like T cells, B cells have to produce highly diverse antigen receptors. The equivalent to the TCR in the B cell is the antibody. An antibody is a large protein with an extremely variable antigen-binding part. B cells have to rearrange gene fragments to form a gene for a suitable antibody, with affinity for only one specific antigen. As opposed to T cells, B cells do not require the context of MHC for recognition of an antigen. Activation of a mature B cell requires binding to a T helper cell that provides cytokine support, in addition to recognition of the right antigen. Thereafter, the B cell undergoes clonal selection, expands into a plasma cell and starts producing vast quantities of antibodies.

**Cytokines**

Cytokines are small cell-signaling peptides and proteins crucial for development, differentiation and activation of the immune cells, but also for their down-regulation. Cytokines can serve as growth factors (such as the granulocyte, macrophage or granulocyte-macrophage colony-stimulating factors), be involved in chemotaxis (Interleukin [IL]-8), or in macrophage activation (Interferon [IFN]-γ). Some cytokines are predominantly involved in activation of T cells (IL-2, IL-7, IL-9, IL-12, and IL-15) and others in activation of B cells (IL-4, IL-7, IL-13, and IL-14). Pro-inflammatory cytokines are IL-1β, IL-6, and tumor necrosis factor (TNF)-α. Cytokines bind to their corresponding cytokine receptors; for instance, the receptor for IL-2 is soluble CD25. Evidently, cytokines and their receptors provide potential targets for treatment of immunodeficiencies and inflammatory diseases.

As we have seen above, the immune cells harbor an enormous capacity for proliferation and expansion, both during maturation and as mature cells. However, the vast majority of mature B and T cells never encounter that specific antigen that they need for activation during their short life-span of a few days. New cells are continuously under production; the turn-over of a blood marrow with the approximate weight of 1.5 kg is two weeks. In a lifetime we produce three tons of hematopoietic tissue.

But what happens to the vast amount of clones that do not produce the TCR or antibody with highest affinity? In order not to obstruct the system, they need to be eliminated in a manner as fast and efficient as they were produced. This happens through apoptosis.
**Mechanisms of apoptosis**

To maintain tissue equilibrium - homeostasis - in multi-cellular organisms, balance of cell proliferation and cell death is required. A human body of 70 kg consists of \( 1 \times 10^{14} \) cells in a state of constant turn-over. Each day we gain 1.2 kg of cells through mitosis, and each day the same amount of old, damaged or superfluous cells needs to be eliminated.\(^{20}\) Some cell types have a more rapid turn-over, such as the epithelial cells in the mucosa of the gastrointestinal tract, or keratinocytes in the skin and hair. As we have already seen, the cells in our blood marrow have a very high capacity for proliferation.

Cell death can be achieved by two principally different means; by programmed cell death, apoptosis, or by its non-physiological counterpart, necrosis.

Necrosis is a pathological type of cell death, causing a rupture of the plasma membrane, organelle destruction and random DNA fragmentation. Release of intracellular content leads to the hallmark of necrosis – inflammation.\(^{21}\) As a contrast, apoptosis can be either physiological or pathological. The apoptotic cell undergoes nuclear condensation and fragmentation, breaks up and forms apoptotic bodies, i.e., particles surrounded by plasma membrane that contain intact organelles and intranucleosomal fragments. These apoptotic bodies are recognized, phagocytosed, and degraded by macrophages and other neighboring cells. Importantly, apoptosis does not result in inflammation, probably due to the intact membrane that hinders noxious particles to reach the surrounding tissue. However, apoptosis requires energy in its demand of RNA and protein synthesis.\(^{21,22}\)

By necessity, cells with a capacity for rapid proliferation also must have a high sensitivity to the mechanisms of programmed cell death. Should mechanisms of apoptosis in the immune system be impaired, a damaged infectious defense and a hyperinflammatory state would ensue.

**Figure 2. Tissue homeostasis**

![Tissue homeostasis diagram](image)
There are several principal pathways that lead to apoptosis. The extrinsic (receptor-mediated) pathway is fundamental to tissue homeostasis, especially for cells in the immune system. The intrinsic (mitochondria-mediated) pathway of apoptosis is typically triggered by cellular stress, such as chemo- and radiotherapy. CTLs and NK cells induce apoptosis either through the extrinsic pathway or via the caspase-independent (perforin/granzyme B mediated) pathway. The common denominator for these elaborately regulated pathways is that they trigger an intracellular cascade of caspase activation. Caspases are proteases that in their cleaved forms become active and effectuate the apoptotic machinery. The leading character in the caspase interplay is caspase 3, the main effector and cleaver of cell proteins. The different pathways converge at the execution phase of apoptosis and result in calcium efflux leading to cell shrinkage, in chromative degradation leading to nuclear fragmentation, in cleavage of integrins leading to loss of cell-to-cell-contact, and in exposure of phosphatidylserine leading to phagocytosis of the apoptotic cell.

Maybe equally important as the process of programmed cell death is programmed cell clearance, i.e., the removal of apoptotic cells. The apoptotic cell externalizes phosphatidylserine on its cell membrane, an “eat-me-signal” required for cell clearance. Dendritic cells and monocyte-derived macrophages carry an array of phagocytosis receptors, such as the integrin receptors, for the uptake of apoptotic cells. After the ingestion of an apoptotic cell they may further down-regulate inflammation by secretion of anti-inflammatory cytokines. Non-cleared apoptotic cells will undergo necrosis, and autoimmunity and inflammation may follow.

In this tremendously complex machinery, all steps and components in priming, triggering, progression, cell death and cell clearance need to function. There are several enhancing or inhibiting substances for apoptosis; endogenous inhibitors worth mentioning in this context are the inhibitor of apoptosis-proteins (IAPs), such as the X-linked IAP (XIAP). This protein binds to and inhibits the initiator caspase-9 as well as the effector caspase-3.

With all this in mind, we can conclude that missing or malfunctioning components of the apoptotic or cell clearance machineries may result in a defunct tissue homeostasis, an impaired tumor surveillance, a compromised viral defence, and that defective elimination of autoreactive T or B cells, or of apoptotic cells carrying autoantigens on their surface, may lead to tissue destruction and autoimmune disease.

**NK cell and CTL mediated induction of apoptosis**

NK cells and CTL carry proteins in vehicles named lysosomes. These lysosomes contain hydrolases, which function in the degradation of intracellular proteins. However, they also contain secretory products, functioning at normal pH, such as perforin and granzyme A/B. Should the cell be activated by the encounter of its specific antigen in combination with an MHC class I-molecule on a target cell, a dramatic reorganization of the cell occurs. The microtubule-organizing center (MTOC), the point from where the microtubules of the cytoskeleton in the cell grow, start migrating towards the point of contact with the target cell, thus polarizing the cell in that direction. The secretory lysosomes, also named lytic granules, start moving along the microtubules towards the synaptic cleft, where they dock at the plasma membrane. A priming
step is required to enable the membrane of secretory granules to fuse with the membrane, whereafter the noxious substances are released. Once exocytosed, perforin forms a pore in the target cell membrane, allowing granzymes to enter and, by cleavage of substrates, induce apoptosis (Figure 3).³²

There are other routes for apoptosis via CTL and NK cells. The secretory lysosomes also contain Fas-ligand that – once exocytosed – cross-links to the Fas receptor on the target cell, activating the extrinsic pathway of apoptosis.³⁴

Obvoiously, deficiencies in these highly regulated mechanisms are likely to result in disease. The most common congenital disease which harbors these deficiencies is the genetic form of hemophagocytic lymphohistiocytosis, the focus of this thesis.
Figure 3a. Resting NK cell or CTL. MTOC=microtubule-organizing center.

Figure 3b. Secretory lysosome exocytosis. Upon encounter between the Fas or the T cell/NK receptor and the corresponding ligand on a target cell, a chain of events is triggered. The NK cell or CTL is activated, polarized, and lysosomes containing perforin and granzyme A/B migrate toward the synaptic cleft, where they dock at the cell membrane. The lysosomes are primed, and eventually undergo fusion with the cell membrane upon which they release their content through exocytosis.32,33
Some concepts of epidemiology

Epidemiology is the medical science that quantitatively studies the distribution and determinants of disease frequency within populations.\(^{35}\) For practical reasons samples of the population need to be studied. Some examples of study types are:

- **Randomized studies:** In this type of trial, patients are randomly allocated to treatment groups. The golden standard for a clinical treatment study is the prospective, double-blinded, placebo-controlled, randomized study.

- **Cohort studies:** A group of individuals is followed or traced over a period of time and studied with regard to certain interventions or risk factors.

- **Descriptive studies:** Observational studies, e.g. case-reports or incidence studies

**Bias** is a systematic error in data. Systematic errors remain even in infinitively large populations. Bias may result from an error in how the studied subjects are selected, *selection bias*, or from misclassification at the level of exposure which leads to *information bias*. Information bias may be the result of recall bias among interviewed subjects, lack of data, faulty measurements, or withdrawal of subjects from the study. *Confounding*, mixing of effects, is another type of systematic error. A confounder is an independent risk factor for the outcome that is also associated with, but not the effect of, the exposure. Confounding is therefore the distortion of a risk factor by the presence of another. Possible methods to deal with confounding are through stratification or by use of regression models. A sub-type of confounding is *confounding by indication*: patients who receive a certain medication usually differ from those who do not. They may have a more severe disease or other risk factors, introducing bias in the comparison of groups.

**Random error:** This is non-systematic error, and thus what remains if all bias would be eliminated. Random error represents the variability in the data that we cannot readily explain. Confidence intervals (CI) are used to indicate the amount of random error in the estimate. The \(p\) value, similarly, indicates the probability that the data align with the null hypothesis \((p = 1.0)\).\(^{36}\)

**Internal validity:** Truth within the study. A study is valid if it represents the truth for the population studied, and results are not distorted by systematic or random error.

**External validity** (=generalizability): Truth beyond the study. Results from a study represent the truth also in other study populations.
Project-specific background

Classification

The term hemophagocytic lymphohistiocytosis (HLH) provides an umbrella under which many different subtypes of the syndrome reside. HLH is traditionally mainly divided into familial, hereditary cases, FHL, and into secondary, acquired cases, sHLH. However, this distinction may not be as clear as it may seem, since both FHL and sHLH usually are triggered by infections and clinical differentiation between the two at disease onset virtually is impossible.

Familial hemophagocytic lymphohistiocytosis (FHL)

Patients with inherited HLH are categorized in this group. As to date, five subtypes of FHL have been described (FHL1-5). Onset of FHL is often, but not always, triggered by an infection.

Other inherited hemophagocytic syndromes.

In addition to the five FHL subtypes, there are other defined inherited syndromes that may give rise to HLH. These syndromes are the Griscelli syndrome type II (GS2), the X-linked lymphoproliferative syndrome type I and II (XLP-1 and XLP-2), the Chédiak-Higashi syndrome (CHS), and the Hermansky-Pudlak syndrome type II (HPS2). To which extent these inherited hemophagocytic syndromes should be classified separately from FHL is a current topic for debate. The pathogenesis of these syndromes is - as we shall see - closely related to that of FHL, involving cytolytic granule membrane transport and secretion and/or affecting apoptosis-induction in target cells.

Secondary hemophagocytic lymphohistiocytosis (sHLH)

Secondary HLH in turn may refer to a number of more distinct subtypes. Secondary HLH may arise following:

- Infection. Many kinds of infections may give rise to sHLH (Infection-associated hemophagocytic syndrome [IAHS]).

Viral infections (Virus-associated HLH, VAHS): The most common infectious trigger, particularly in patients of South-East Asian descent, is the Epstein Barr-virus (EBV). EBV is a virus of the herpes group causing mononucleosis, and >90% of adults have developed immunity. EBV infection is typically subclinical and/or uncomplicated, but it is known that it may induce lymphoproliferative acute and chronic EBV-infections in severely immunocompromised patients or patients with innate immune defects. Why Asian patients seem to be more prone to develop EBV-HLH is unclear, and it is feasible that these patients have an underlying genetic predisposition yet to be identified. Other viruses from the herpes group known to be triggers of HLH are cytomegalovirus (CMV), humane herpes virus type 6, parovirus B19 and varicella zoster virus. However, a number of other viral infections have been described to trigger HLH, including HIV. It is known that influenza may give rise to HLH, and maybe in particular...
some strains, such as the pandemic 2009 influenza H1N1. Drastic improvement of H1N1-induced HLH with HLH-94 treatment has been reported.

_Bacterial infections_ of several types have also been described as triggers of HLH. However, severe septicemia, in particular among neonates, may be difficult to differentiate from the clinical picture of infectious-triggered HLH.56,57

_Parasitic infections_, predominantly leishmania, can induce a fulminant HLH-like picture, but HLH has also been described with other parasitic diseases, e.g. malaria.53,58-60

- **Systemic rheumatologic disease.** The most common rheumatic disease associated with HLH appears to be systemic onset juvenile idiopathic arthritis (SoJIA), but systemic lupus erythematosus, adult onset Still’s disease, Kawasaki disease,61 and other diseases have also been described.62-64 Rheuma-associated HLH is often referred to as macrophage activating syndrome (MAS) by rheumatologists. Initiating factors may be triggering infections or the combination of the rheumatic disease and treatment, such as with immunosuppressive agents. There are different definitions of MAS, such as for SoJIA-MAS and SLE-MAS, and they in turn differ somewhat from that of HLH.65,66 However, there is evidence that rheuma-associated HLH and MAS may be the same disease entity, with reduced NK cell function, decreased SAP expression and/or reduced perforin expression, only viewed from different disciplinary perspectives.67-71

- **Malignancy.** HLH may occur in patients with certain malignancies, in particular leukemias and lymphomas, and may then be referred to as malignancy-associated hemophagocytic syndrome, (MAHS).72-74

- **HLH has lately been recognized as a syndrome that may develop in severely ill patients of varying ages in intensive care units.** It may be that the conditions of sepsis, systemic inflammatory response syndrome, multi-organ system failure and HLH form a continuum of immune dysregulation in the presence of a trigger, and that HLH may develop in individuals with subtle immunological defects at times of increased physiological stress.78

**Genetics**

FHL is autosomal recessive in inheritance. For an overview of the different genes described for FHL and inherited hemophagocytic syndromes, see Table 1.

*Genes involved in FHL*

There are 5 reported locuses and 4 identified genes for FHL. The locus for FHL1 was identified by homozygosity mapping by Ohadi _et al_ in 1999. However, the responsible gene remains unidentified, and only a few patients have been linked to the locus. In 1999, Stepp _et al_ identified the first disease-causing gene for FHL, the PRFI gene (FHL2), and since then a
further three disease-causing genes have been identified: \textit{UNC13-D (FHL3)}, \textsuperscript{81} \textit{STX11 (FHL4)}, \textsuperscript{82} and \textit{STXBP2 (FHL5)}.\textsuperscript{83,84}

\textbf{Genes involved in the other inherited HLH-associated syndromes}

Griscelli syndrome type II is caused by mutations in the \textit{RAB27A} gene,\textsuperscript{85} CHS by mutations in the \textit{LYST} gene,\textsuperscript{86,87} HPS2 in the \textit{AP3B1} gene,\textsuperscript{88} XLP1 in the \textit{SAP} (Signaling lymphocyte activation molecule [SLAM]-associated protein) gene, also known as \textit{SH2D1A},\textsuperscript{89} and XLP2 in the \textit{XIAP} gene.\textsuperscript{90} The inheritance is autosomal recessive in GS2, CHS and HPS2, but X-linked in XLP1 and XLP2. Therefore, XLP1 and XLP2 only affect boys.

\textbf{Genotype-phenotype studies}

Evidently, the type of mutation is important for the phenotype of FHL, but there is an extensive genetic and allelic heterogeneity of the disease. In addition, it has been demonstrated that the phenotype, at least in mice, may depend on the infectious trigger.\textsuperscript{91} Several different mutations within the above-mentioned genes have been reported, both \textit{missense} mutations (point mutations in the DNA sequence resulting in production of a different amino acid) and \textit{nonsense} mutations (point mutations giving rise to a non-functional protein product). As could be expected, missense mutations have been shown to be more likely to give rise to a later onset and may also be associated with residual cytotoxic function.\textsuperscript{92} FHL patients have not only been reported to carry homozygous mutations (mutations on both gene alleles), but also heterozygous mutations (mutations on only one allele).\textsuperscript{93-96} Heterozygosity may be associated with a later onset of disease, or a milder or subclinical form,\textsuperscript{95} but it does not rule out a severe primary immunodeficiency.\textsuperscript{96} Obviously, some of these patients may have compound heterozygous mutations with only one mutation detected. This later notion is supported by recent findings by Meeths \textit{et al}, who found previously undetected additional genetic aberrations in \textit{UNC13D} in a cohort of patients with previously only monoallelic mutations identified.\textsuperscript{97} Functional cell studies on first degree relatives to FHL patients have shown an impaired or even absent NK cell function in obligate carrier parents, but also values within the normal range.\textsuperscript{98}

Genotype-phenotype studies show that FHL2 patients may be younger at onset\textsuperscript{99,100} and, although FHL2 patients have been reported from various parts of the world, they seem to be more common in Turkish, Middle Eastern, African American and Japanese ethnic groups.\textsuperscript{92,99,101} FHL2 is rare among patients of Scandinavian or German descent. Recently, it has been shown that a deep intronic \textit{UNC13D} mutation is the most common aberration in Swedish infants, and that it is spread also in the rest of Scandinavia. Furthermore, an intronic \textit{UNC13D} point mutation seems to account for a proportion of FHL3 among Caucasians throughout Europe.\textsuperscript{97} FHL3 patients have been reported to have a higher frequency of CNS involvement.\textsuperscript{100,102} FHL4 seems to be most common in Turkish patients,\textsuperscript{99} and has not yet been described in Japanese patients.\textsuperscript{103} FHL5 patients have a highly variable disease severity, and symptoms not typically associated to HLH, such as colitis, bleeding disorders, and hypogammaglobulinemia have been reported in about a third of the patients, respectively.\textsuperscript{96} Depending on the ethnic origin, FHL2-4 is reported to account for about 30-70\% of the patients.\textsuperscript{104} According to a French study,\textsuperscript{84} only 10\% of their cohort of FHL patients now lack a genetic diagnosis.
Table 1. Genes involved in FHL and HLH-associated syndromes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Gene location</th>
<th>Protein function</th>
<th>Reported by</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHL1</td>
<td>Unknown</td>
<td>9q21.3-3.22</td>
<td>Unknown</td>
<td>Ohadi et al, 1999</td>
</tr>
<tr>
<td>FHL2</td>
<td>PRF1</td>
<td>10q21-22</td>
<td>Pore forming, involved in target cell cytolysis</td>
<td>Stepp et al, 1999</td>
</tr>
<tr>
<td>FHL4</td>
<td>STX11</td>
<td>6q24</td>
<td>Vesicle intracellular trafficking/membrane fusion</td>
<td>zur Stadt et al, 2005</td>
</tr>
<tr>
<td>FHL5</td>
<td>STXBP2/UNC18B</td>
<td>19p13.2</td>
<td>Co-localization with syntaxin-11, stability of both proteins</td>
<td>zur Stadt et al, 2009; Côte et al, 2009</td>
</tr>
<tr>
<td>XLP1</td>
<td>SAP/SH2D1A</td>
<td>Xq25</td>
<td>Signal transduction in NK and CTL/vesicle trafficking</td>
<td>Coffey et al, 1998</td>
</tr>
<tr>
<td>XLP2</td>
<td>XIP/BIRC4</td>
<td>Xq25</td>
<td>Inhibition of caspases, the effectors of apoptosis</td>
<td>Rigaud et al, 2006</td>
</tr>
<tr>
<td>GS2</td>
<td>RAB27A</td>
<td>15q15-21.1</td>
<td>Tethering/docking of secretory granules</td>
<td>Ménasché et al, 2000</td>
</tr>
<tr>
<td>CHS</td>
<td>LYST</td>
<td>1q42.1-42.2</td>
<td>Sorting of endosomal proteins into late endosomes, lysosomal fission</td>
<td>Barbosa et al, 1997; Perou et al, 1997</td>
</tr>
<tr>
<td>HPS2</td>
<td>AP3B1</td>
<td>5q14.1</td>
<td>Cytolytic lysosomal movement, sorting of granule proteins</td>
<td>Dell’Angelica et al, 1999</td>
</tr>
</tbody>
</table>

Animal models
By inactivating certain genes in animals, so called “knockout mice” can be created to provide a model for a disease. Murine models for FHL are available for FHL2 and 3, and there are also models for GS2, CHS, HPS2,105 XLP1 and XLP2,106 facilitating studies of the traits in these diseases. Interestingly, humanized mice transplanted with human CD34+ cells, may be used as animal models of EBV-induced HLH.107 However, mice aren’t men. We can never fully trust that results in animal experiments are valid also in humans. FHL-patients serve as natural, “human knock-outs”. Through clinical studies performed with the help of these patients, we may learn more about basic biological mechanisms in humans.
Pathogenesis and pathophysiology

FHL and inherited hemophagocytic syndromes
Description of the genetic aberrations in FHL and hereditary HLH-associated syndromes, and subsequent in-vitro and in-vivo studies of knockout mice, has resulted in deepened insights into the pathogenesis of HLH, and also into mechanisms of cytotoxicity in normal immune cells. During secretory lysosomal exocytosis of immune cells and subsequent target apoptosis, a number of proteins need to perform their action in a functional manner. Table 1 and Figure 4 provide an overview of the proposed mechanisms of actions of the gene products for FHL-causing genes, but also for the genes involved in GS2, XLP1/2, CHS and HPS2.

Non-immunological symptoms
However, not only NK cells and T cells are dependent on lysosomal transport and exocytosis. Other cells with secretory lysosomes are platelets, granulocytes, mast cells, melanocytes and neuronal cells, among others. This explains some of the clinical characteristics in other hereditary HLH syndromes, and perhaps also in FHL5.

RAB27A, LYST, and AP3B1 are all expressed in melanocytes. Defect melanosomal transport, and subsequent lack of exocytosis of melatonin from melanocytes to keratinocytes, explains the link to albinism in GS2 and CHS patients. In HPS2, the albinism seems to be a coincidental, since AP3B1 is also required for the formation of melanin. The LYST of CHS is also expressed in neurons, and LYST and AP3B1 are expressed in platelets. Defect signaling between neurons could therefore be important in progressive neurodegeneration in CHS, and defects of dense granule biogenesis in platelets could cause bleedings in HPS2 and CHS patients.

Secondary HLH
The pathogenetic mechanisms in sHLH have not been clearly delineated; however, one can speculate that these patients also harbor an immunosusceptibility associated to NK and CTL function. More insights into the etiology of sHLH could give potential targets for novel therapeutic interventions.

The role of cytokines
HLH is thus caused by defect down-regulation and concurrent auto-stimulation of the immune response. This hyperinflammatory state creates hypercytokinemia within the body. Included in this “cytokine storm” are IFN-γ, IL-1, IL-6 and TNF-α, all known to be highly elevated in patients with HLH. This may not be surprising, since active macrophages secrete TNF-α and IL-6, and activated CTL produce IFN-γ. Jordan et al in 2004 presented findings from cytokine neutralization of IFN-γ, demonstrating that IFN-γ not only seems to drive inflammation further, but that it was uniquely essential for HLH development in mice. IL-2 is a potent mitogen of activated CTL, important for CTL auto-stimulation and clonal expansion. It is also elevated in patients with HLH, as are levels of the alpha-chain of its receptor, sCD25. The role of different cytokines needs further elucidation, since this may provide important clues for possible treatment strategies.
Figure 4. **Pathogenesis of FHL and other familial HLH syndromes.** Perforin forms a pore in the target cell, allowing granzymes and other noxious substances to enter into the target cytoplasm, where they trigger apoptosis. The β subunit of AP3 and SAP are involved in the movement of granules along the microtubules. Maturation of lysosomes and docking at the immunological synapse is impaired in deficiency of lysosomal trafficking regulator-deficiency. RAB27A is a GTP-binding protein essential for membrane transport and tethering to the plasma membrane. Munc13-4 is thought to mediate priming of the granules for fusion with the membrane, and syntaxin 11 and syntaxin-binding protein 2 are also important for fusion. In the target cell, X-linked inhibitor of apoptosis binds to and inhibits caspase 3, 7 and 9, effectors of the apoptotic machinery.
AIMS OF THE THESIS

The general purpose of this thesis was to improve survival and reduce morbidity in children suffering from hemophagocytic lymphohistiocytosis and other familial hemophagocytic diseases, by studying treatment outcomes, clinical presentation and risk factors of the disease. The specific aims were:

1. To provide a detailed, long-term analysis of the outcome of the HLH-94 treatment protocol for hemophagocytic lymphohistiocytosis (Paper I)

2. To study clinical characteristics at onset of disease in all patients, and to evaluate if and how these differ between sub-groups of patients (Paper I)

3. To study the frequency and manifestations of HLH in the central nervous system in the acute phase, to describe neurological late effects in survivors, and to investigate if and to what extent acute CNS-disease is a risk factor of survival and late effects (Paper II)

4. To present treatment results for patients with other inherited hemophagocytic syndromes; X-linked lymphoproliferative syndrome, Chédiak-Higashi syndrome and Griscelli syndrome, treated by HLH therapy (Paper III)

5. To investigate possible clinical prognostic parameters in children with HLH, and to study if one or a combination of these could be used in risk stratification (Papers IV and V)
MATERIALS AND METHODS
Data Collection

For papers I-III and V, data were collected from the HLH-94 or HLH-2004 treatment study databases. The data collections for these treatment studies were made by study-specific forms completed by the treating clinician and collected by the local treatment centers, from where they were delivered to the data study center in Stockholm. These forms were to be completed at onset of disease, two and six months into HLH therapy, and then yearly. For patients who received transplants, data were reported pre-transplant, 100 days thereafter, and then once yearly post-transplant. In addition, the HLH-2004 study included specific forms with in-depth information on primary diagnostic criteria, serious adverse events and mortality report forms. Furthermore, information on results of genetic analyses, and of laboratory parameters at two and four weeks into initial therapy was requested, as well as a more specific assessment of neurological symptoms and signs.

For paper III, additional information on how diagnosis was obtained, as well as information on disease state and survival at last follow-up, was collected through direct contact with the treating clinicians. The reason for this was that follow-up was not necessarily being requested within the treatment studies after diagnosis of another form of hemophagocytic disease.

For paper IV, data was obtained via a separate questionnaire. This was sent out to the participating center coordinators, who requested the data from the treating hospitals. In an effort to collect as complete data as possible, a physician in Scandinavia and one in Italy went to the different treating hospitals in order to directly collect missing data.

Study populations

Paper I
This study included prospective data from 249 patients treated by the HLH-94 protocol. The patients were <16 years of age before start of therapy, fulfilled all diagnostic criteria, or had a familial disease, as defined by an affected sibling, in combination with a clinical picture suggestive of HLH. Patients were excluded if they had another defined underlying disease or malignancy, or if they had received previous cytotoxic or cyclosporin A (CSA) therapy. They started HLH therapy between July 1st 1994 and December 31st, 2003, and date for latest data entry was October 2008.

Paper II
This study included 193 patients recruited from the HLH-94 treatment data base. They were <16 years of age, fulfilled all diagnostic criteria, or had a familial disease, as defined by an affected sibling, in combination with a clinical picture suggestive of HLH. Patients were excluded if they had another defined underlying disease or malignancy, or if they had received
previous cytotoxic or CSA therapy. Furthermore, treatment or intention to treat by the HLH-94 protocol was prior to July 1st, 2003, and all included patients had to have information on neurological symptoms as well as on CSF cell counts and/or protein levels at diagnosis. For inclusion in the evaluation of neurological late effects, a neurological follow-up of >1 year was required. Date of last data entry was April 2005.

Paper III
This was a report on nine patients enrolled in the HLH-94 or HLH-2004 treatment databases, aged <16 years and who started therapy prior to December 31st, 2004, and who were retrospectively identified to have an inherited HLH-associated syndrome (GS2, n=5, XLP, n=2, or CHS, n=2).

Paper IV
This study included data from 232 patients reported from three European centers participating in the CureHLH consortium, which was a collaborative research effort supported by the European Union. The evaluated patients started treatment according to the HLH-94 or the HLH-2004 protocol prior to January 2009, were <19 years of age, fulfilled HLH diagnostic criteria and/or had a familial and/or a genetically confirmed disease. Patients with previous cytotoxic therapy and/or another underlying disease were excluded.

Paper V
As in paper IV, patients included in this study were <19 years of age, fulfilled HLH diagnostic criteria and/or had a familial and/or a genetically confirmed disease. They had received no previous cytotoxic therapy and had no other underlying disease. In this study, 297 patients were recruited from the HLH-2004 treatment database and the date of last data entry was July 2011. For certain analyses of this study, the patients that were also included in paper IV (n=70) were excluded, as detailed later, leaving 227 patients eligible for analysis.

Definitions
In paper I-II and IV-V, the inclusion criteria were chosen with the intent to include only patients with HLH, and no other less well-defined HLH-resembling syndromes. Therefore, patients had to fulfill all HLH criteria or have an affected sibling. For papers IV and V, a known HLH-causing mutation was also accepted as an inclusion criterion. With these rather strict inclusion criteria, we may have excluded patients with secondary or less advanced disease, which should be considered in interpretation of survival data.

Familial HLH was defined as the presence of an affected sibling in paper I and II. In HLH-94, the database from which these patients were recruited, there was no information of genetic subtypes of HLH, since the first FHL-causing gene was first described 5 years after the study.
was launched. Still when papers IV and V were written, only a proportion of patients could be identified by genetic studies, which is the reason why patients in these papers were considered as familial not only after genetic diagnosis, but also based on presence of an affected sibling.

Secondary HLH: Patients that fulfill diagnostic criteria but lack a history of familial disease or a known disease-causing mutation can either have a familial or a secondary HLH. We know that both patients with FHL and sHLH can have severe disease, and that there is no way to clinically securely separate in between these two groups at presentation. Based on our experience of treatment response, patients who had been able to stop all HLH therapy for >1 year without needing a HSCT, and who had had no signs of disease reactivation were presumed to be sHLH patients. Although unsatisfactory, this is the definition also used in the previous study of HLH-94 treatment outcome.119

CNS-disease in HLH has not been finally defined. In paper II we defined CNS disease as presence of neurological symptoms and signs, or elevated values of CSF cells and/or proteins. A pathological brain imaging was not included in this definition, since only a minority of patients had a computed tomography (CT) scan or magnetic resonance imaging (MRI) available prior to therapy.

Disease activity: In the HLH-94 and HLH-2004 treatment protocols, patients with HLH are considered to have clinically active disease if one or more of the following criteria persist: fever, cytopenia, hepatosplenomegaly and clinical signs of active CNS disease. For complete disease resolution, normalization of serum transaminases, triglycerides, fibrinogen, ferritin, and CSF protein and cell counts are required. However, in the HLH-94 and HLH-2004 follow-up forms, the clinicians stated presence of disease activity with a “yes” or a “no”.

Statistical analyses

Data was collected in databases, and statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS), version 11.5 or 17.0 (Chicago, IL, USA).

Univariate relations were examined by use of the Chi-square test or, where frequencies were small, the Fisher’s exact test. The Mann-Whitney U test was used for comparison of differences in median time and age in paper I. Probability of survival was estimated using the Kaplan-Meier life table method in papers I and II, and univariate comparison of survival between groups was made by the Log rank test. Maximum follow-up after start of therapy was used in paper I and paper II, or in paper I - from the date of HSCT in patients who received transplants. For multivariate analysis, Cox proportional hazards regression was performed in papers II, IV and V. In paper II, maximum follow-up time was used, but in papers IV and V an outcome right truncation at four months after therapy start was used and, since the aim was
to study pre-transplant death, patients were censored at last follow-up or HSCT. The rationale behind the outcome truncation was to select patients who were in need of an early transplant, and to avoid including patients deceased due to an unsuccessful or tardy donor search. Risks calculated by Cox regression were presented as crude and adjusted hazard ratios (HR:s), with adjustment for CNS disease group in paper II, as well as for HSCT, sex and age. In papers IV and V, HR:s were adjusted for sex, age, and for the other statistically significant predictors identified from the same time-point. Throughout, results were presented with 95% confidence intervals (CI:s), and a two-sided $p$-value of $<0.05$ was considered statistically significant.
RESULTS
AND
DISCUSSION
Epidemiology

Incidence

There is only one published incidence study of HLH. In 1991, Henter et al reported that the incidence in Sweden between 1971 and 1986 could be estimated to 1.2/1,000,000 children/year, or 1/50,000 living newborns. This is somewhat lower than the incidence of aplastic anemia in the Nordic countries (1.95/1,000,000 children/year), and slightly higher than the incidence of severe combined immunodeficiency (1/71,000 living newborns). Recognizing and diagnosing HLH may be difficult, and since awareness of HLH has increased since the publication of this incidence study it is likely that more children are correctly diagnosed today than at the time of that incidence study. Furthermore, the hereditary disease HLH is likely to be more common in countries where consanguineous marriages are frequent.

The incidences of GS2, CHS and HPS2 are unknown. HPS2 patients who have developed picture of HLH are only known through a few case reports. For XLP, an international registry was established in 1980 by Purtilo and Grierson, which 15 years later included 272 affected members from 80 kindreds. The incidence is estimated to 1-3/1,000,000 men born.

Sex distribution

A slight male preponderance of patients with HLH has previously been reported. In paper I, we found a male proportion of 55%. However, this difference was not statistically significant (95% CI of 49-61%). Male proportions were similar (55%, 55%, and 54%) in papers II, IV and V. It is difficult to explain a male predominance in a disease of autosomal inheritance: a factual difference could depend on sex-linked genetic subtypes of FHL not yet described, or inclusion of patients with XLP who have been misdiagnosed. In the latter case, the sex difference should diminish in future reports as XLP today is a known and important differential diagnosis for boys with (especially EBV-associated) HLH.

Furthermore, in paper I we found that female sex was more common in the subgroup that survived after all therapy termination and without a HSCT, i.e., presumably secondary patients, as compared to in the remaining patients (61% as compared to 45%). An equal difference in sex distribution in patients with EBV-associated sHLH has previously been reported in a study from Japan. The reason for this is unclear: to our knowledge, there are no known sex differences in the vulnerability or response to EBV infection, and XLP, the immunodeficiency known for susceptibility to EBV-infection, is X-linked in inheritance. However, new candidate genes with autosomal recessive inheritance may represent a proportion of the patients of EBV-induced lymphoproliferative immunodeficiencies.
**Age at onset**

The median age at onset of symptoms has been reported to be three months, and symptoms develop before 1 year of age in 70-80% of children with HLH.\textsuperscript{127,129} Neonatal or even antenatal onset has been reported, and may have been the cause of prematurity.\textsuperscript{54,130-133} In sHLH, onset typically occurs later in childhood.\textsuperscript{128} In line with these findings, we found an overall median age at onset of 8 months, (range 0-184) and a median age of 24 months (range 2-184), in patients with sHLH in paper I.

However - and importantly - age alone does not reliably discriminate between sHLH and FHL. In paper I, the oldest patient with familial disease was 12 years at onset. We know that patients with genetically confirmed FHL may have a late onset,\textsuperscript{92,93,96} but also that the opposite may be true: bacterial-associated sHLH has been reported in an extremely premature infant,\textsuperscript{54} and in paper I, the youngest sHLH patient was only 2 months of age at start of therapy.

HLH is being increasingly reported in adults.\textsuperscript{93,134-137} Furthermore, there are interesting indications from intensive care medicine that patients of any age can develop an HLH-like picture as part of a fulminant septicemia, multi-organ failure, or shock.\textsuperscript{77,138}

Patients with the other inherited hemophagocytic syndromes are also young at onset of their HLH symptoms; XLP patients have an average onset age of 2.5 years\textsuperscript{89} and GS2 patients a median age of 1.5 years at onset.\textsuperscript{19,139} For patients with CHS, a wide age variation at disease acceleration is seen, and some patients seem not to develop HLH at all.\textsuperscript{140} In a recent publication, it was postulated that that subtle differences of CTL function in CHS determines susceptibility and age at onset for HLH.\textsuperscript{141} A HPS2 patient has been reported to develop HLH at 3 years of age,\textsuperscript{123} but an additional heterozygous Rab27a mutation may have contributed to the disease. In paper III, the age at onset of the accelerated phase with HLH symptoms in the two XLP patients was 4 and 6 years, in the five GS2 patients it was 0-3 years, and the two CHS patients 5 and 7 years, respectively.

**Demographics**

HLH has been reported from all parts of the world. Presumably, FHL is more common in parts of the world where consanguineous marriages are frequent. In paper I, we report on patients from 25 countries on five different continents: Australia, Argentina, Austria, Canada, the Czech Republic, Denmark, Finland, Germany, Hong Kong, Iceland, Italy, Japan, Korea, the Netherlands, Norway, Oman, South Africa, Saudi Arabia, Spain, Sweden, Switzerland, Turkey, the United Kingdome, the United States of America, and the former Republic of Yugoslavia.
Symptoms and laboratory findings

Clinical features in HLH

Clinical features in patients with HLH arise due to a massive inflammatory response caused by a hypercytokinemia. This in turn causes multi-organ system disease and, if untreated, leads to death. Symptoms may come gradually or develop over the course of a few days. The two most common clinical signs are prolonged fever, which is unresponsive to antibiotics, and hepatosplenomegaly. Other symptoms are jaundice, an uncharacteristic skin rash, lymphadenopathy, edema, failure to thrive, and various neurological symptoms. Symptoms may depend on the disease localization, and neurological symptoms such as seizures have been described as a presenting sign of HLH in the CNS.

Fever is the result of pyrogenic cytokines, such as IL-1, IL-6, TNF-α, but naturally, there may also be a concomitant infection in part responsible for the fever. Hepatospl enomegaly seems to be a direct effect of organ infiltration by lymphocytes and macrophages.

CNS disease is also thought to be caused by tissue infiltration, and is pathologically variable with infiltration of meninges, perivascular infiltrates and, in more severe cases, tissue infiltration as well as multifocal necrosis and edema. Neurological symptoms vary in character and intensity; a range from mild symptoms to seizures, coma, brain stem symptoms, or ataxia have been described, and the frequency of these symptoms has been reported to 26%. Abnormalities on MRI consist of parenchymal atrophy, diffuse abnormal signal intensity in the white matter, focal hyperintense lesions in both white and gray matter, defects of myelination and parenchymal calcification. In paper II, we reported neurological symptoms in 72/193 (37%) patients at onset, the most common symptoms being seizures, meningistic signs, and irritability. Neuroradiological abnormalities (in MRI or CT scan) were reported in 30% of the patients, and included signs of demyelination and white matter lesions. Importantly, a pathological radiological exam was found also in five patients without neurological symptoms or abnormal CSF examination. Assessment of neurological symptoms may be difficult in very young and severely ill children. We therefore believe that the frequency of CNS symptoms may be underestimated.

Laboratory findings in HLH

Laboratory studies typically reveal anemia, neutropenia and thrombocytopenia. These findings have been reported to be, at least in part, an effect of macrophage engulfment in the bone marrow, hemophagocytosis, that in turn is critically dependent on IFN-γ. Interferon-γ is produced by CD8+ T cells stimulated by persistent antigen exposure. Other typical findings are elevated fasting triglycerides and low fibrinogen. Triglycerides are elevated due to inhibition
of lipoprotein lipase by inflammatory cytokines such as TNF-α, IL-6 and IFN-γ [151]. Fibrinogen, an acute phase reactant known to be elevated in chronic inflammation [152], is low in patients with HLH. This is due to secretion of plasminogen activator by macrophages, causing plasmin to cleave fibrinogen [19]. Overall, coagulation is disturbed.

Ferritin is typically highly elevated in patients with HLH [153]. Ferritin, i.e., iron that is bound to the intracellular protein apo-ferritin, is the normal form of storage for body iron. Phagocytic macrophages are thought to be an important source of serum ferritin, and in vitro studies have shown that ferritin accumulates during maturation of macrophages and that macrophages involved in phagocytosis produce ferritin [68]. Furthermore, ferritin is elevated secondary to the up-regulation of heme-oxygenase, a heat shock protein expressed in response to cytokines and endotoxins [154]. Ferritin is an acute phase reactant and as such, elevated values are unspecific in severely ill patients. However, the extremely high levels reported in HLH patients seem both sensitive and specific for the disease [153].

Other common laboratory features of HLH are elevated liver enzymes, (conjugated) bilirubin and lactate dehydrogenase. Albumin typically is low, as is sodium. Laboratory signs of renal failure, such as an elevated creatinine and urea, are less common [127].

Elevated values of proteins and cells in the CSF has been reported to be found in 53-76% of HLH patients [127, 146]. In paper II, we found an abnormal CSF examination in 51% of the patients. Pleocytosis was often mild, and in 77% CSF cells were <20 x10^6/L. Hemophagocytosis was also found within the CSF. A normal clinical neurological status did not preclude CSF abnormalities.

Hemophagocytosis is a frequent finding in the bone marrow, but it may be absent, particularly earlier in the disease course [127]. Repeated examinations may be required. Although informative, functional cell assays such as analyses of NK cell function and measurements of sCD25 (which typically is elevated in HLH), may be expensive, and are only available in specialized laboratories.

In conclusion, all physical symptoms and signs, as well as laboratory parameters commonly abnormal in HLH patients are directly or indirectly caused by the immune deficiency with defect down-regulation of the immune response, expansion of activated T lymphocytes and macrophages, and by the resulting cytokine storm (Table 2).

In paper I, the presenting symptoms and signs of 249 patients treated by HLH-94 therapy were summarized (Paper I, Table 2). Data on variables included in the HLH-94 diagnostic criteria were not included since fulfillment of these was one inclusion criterion for the evaluation, and therefore would be biased. Our data were well in line with previously reported findings in HLH patients, but represented a larger material than previously reported. Furthermore, we showed
how these parameters differed between sub-groups in the material; patients who died within the first initial two months of therapy, patients with presumed sHLH, patients who received transplants, and patients with a familial disease. The group that differed most from the remaining patients was patients with sHLH. They more seldom had symptoms of tissue infiltration, such as CNS disease and hepatomegaly, but more frequently had lymphadenopathy, possibly reflecting the triggering viral infection.

Table 2. Some HLH symptoms and signs, and their pathogenesis.

<table>
<thead>
<tr>
<th>HLH symptom or sign</th>
<th>Caused by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>Pyrogenic cytokines (IL-1, IL-6, TNF-α)</td>
</tr>
<tr>
<td>Hepatosplenomegaly</td>
<td>Organ infiltration by macrophages and leukocytes. Hemolysis, destruction of blood components</td>
</tr>
<tr>
<td>Cytopenia</td>
<td>Consequence of hemophagocytosis, at least in part</td>
</tr>
<tr>
<td>Hemophagocytosis</td>
<td>IFN-γ-driven, persistence of triggering antigen and activated APC</td>
</tr>
<tr>
<td>Elevated triglycerides</td>
<td>Inhibition of lipoprotein lipase by inflammatory cytokines</td>
</tr>
<tr>
<td>Decreased fibrinogen</td>
<td>Following plasminogen activator secretion by macrophages</td>
</tr>
<tr>
<td>Elevated ferritin</td>
<td>Caused by cytokines/endotoxin, produced during monocyte/macrophage activation and phagocytosis</td>
</tr>
<tr>
<td>Elevated sIL-2R (s-CD25)</td>
<td>Produced by activated CTL</td>
</tr>
<tr>
<td>Decreased/absent NK cell and CTL function</td>
<td>The underlying defect in HLH, causing inefficient triggering of the apoptotic machinery</td>
</tr>
<tr>
<td>Elevated liver enzymes</td>
<td>Accumulation of inflammatory cells, mainly lymphocytes, in the liver</td>
</tr>
<tr>
<td>CNS-disease</td>
<td>Accumulation of inflammatory cells, mainly lymphocytes, in the CNS</td>
</tr>
</tbody>
</table>

Additional symptoms in the other inherited hemophagocytic syndromes

The following syndromes can develop an “accelerated phase”, a clinical picture of HLH which cannot be distinguished from other genetic or acquired HLH forms, and which typically is fatal unless treated. Furthermore, the syndromes are characterized by additional symptoms.

- **Griscelli syndrome type II**
  Patients with GS2 have a partial albinism with silvery-white hair, and varying degree of skin hyper- or hypopigmentation, or even “leopard-like areas of skin”, related to the inborn defect of melanosome exocytosis. Examination reveals an accumulation of melanosomes in melanocytes, and large clumps of pigment in the hair shafts visible in light microscope, or even, in the latter case, to the bare eye. GS2 patients also have varying degrees of neutrophil dysfunction. Neurological affection is common in GS2 patients who have developed HLH, and there is currently a debate whether these manifestations result from CNS-HLH or a non-inflammatory degenerative neurologic mechanism.
- **X-linked lymphoproliferative disease**
  XLP is characterized by the triad of increased susceptibility to EBV-infection, acquired hypogammaglobulinemia and lymphoma.\(^{158}\) 57% of patients die of infectious mononucleosis, 29% acquire hypogammaglobulinemia, and around 30% present with lymphoma as the initial manifestation of the disease.\(^{126,159,160}\) Patients often appear healthy prior to encounter of EBV, but then develop fulminant infectious mononucleosis, with polyclonal expansion of T and B cells, which is mortal in 92% of cases. All patients die before their fifth decade of life.\(^{89}\) XLP2 patients do not seem to develop lymphoma, have no degranulation defect in NK cells,\(^{106}\) and furthermore seem to develop less severe HLH.\(^{161}\)

- **Chédiak-Higashi syndrome**
  Like GS2 patients, CHS patients have a partial albinism. Furthermore, they may suffer from photophobia and nystagmus, are susceptible to infections due to few or malfunctioning neutrophils,\(^{162}\) and are at increased risk of developing tumors.\(^{163}\) Development of HLH occurs in 85-90% of the patients.\(^{140}\) CHS patients may develop neurological manifestations of their disease, also many years after a successful HSCT. This may be an effect of steady long-term progression of the lysosomal defects in neurons and glia cells.\(^{111,164}\) Diagnosis can be obtained without genetic results since the patients have characteristic giant lysosomes in granulocytes, lymphocytes and monocytes, easily detectable on blood smear.

- **Hermansky-Pudlak syndrome type II**
  As previously stated, only a few case reports demonstrate the clinical tendency to develop HLH in HPS2 patients. Other features of HPS2 are congenital neutropenia resulting in recurrent bacterial infections, oculocutaneous albinism and absence of dense bodies in platelets, resulting in bleeding.\(^{165}\) Moreover, dysmorphic facial features, microcephaly and mental retardation have been reported.\(^{166,167}\)
Diagnostics

“The eye will not recognize what the mind does not know”
/Confucius

Diagnosis and diagnostic criteria

Diagnosis of HLH may be difficult. The initial presentation may vary: there are case reports of patients who have had neurological manifestations of HLH as the presenting signs, or even reports where the symptoms have been misinterpreted as child abuse. Other children have presented with fulminant hepatic failure, or have been treated for septicemia before coming to the knowledge of pediatric hematologists and/or rheumatologists. The diagnostic criteria for HLH are unspecific taken one by one, but with knowledge about them, the physician may “connect the dots”, and end up in the correct diagnosis.

Clinical diagnostic criteria for HLH were first proposed in 1991, and these were used in the subsequent HLH-94 treatment study (Paper I). In an effort to increase specificity, the criteria were revised in 2004. The 1991 and current diagnostic criteria for HLH are presented in Table 3.

For patients with MAS slightly different diagnostic approaches have been proposed, including falling platelet and leukocyte counts, hyperferritinemia, hemophagocytosis, fever, increased liver enzymes, hypertriglyceridemia and hypofibrinogenemia.

In papers I, II, IV and V, patients who did not have familial disease were included only if they fulfilled the HLH diagnostic criteria of their respective protocols. This makes an evaluation of the frequency of diagnostic criteria impossible within these cohorts.

Differential diagnosis

There are several differential diagnoses of HLH. In addition to different kinds of secondary HLH and the other inherited syndromes that may develop HLH, presented earlier in this thesis, HLH-like symptoms may arise in Kikuchi’s disease, or be a complication of Langerhans cell histiocytosis. Other differential diagnoses include Omenn syndrome, lysinuric protein intolerance, severe combined immunodeficiency and DiGeorge syndrome, as well as numerous other conditions.
As is the case in most disorders, an early and accurate diagnosis is the most important factor for a prompt start of an effective therapy. In the latter decades, HLH has become a more known disease entity, in particular among pediatric hematologists and rheumatologists. However, HLH is important to have in mind as a diagnostic possibility for pediatricians in many other subspecialties; the first to encounter these patients may be specialists in infectious medicine, hepatologists/gastroenterologists, neurologists, or specialists in intensive care and neonatology units. Diagnostic and therapeutic delay is prone to have dire effects on survival for patients with HLH.

Table 3. Diagnostic criteria for HLH.

<table>
<thead>
<tr>
<th>Symptom / Sign*</th>
<th>1991: The 5 first criteria required</th>
<th>2004: 5/8 criteria Required</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical criteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Fever</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Splenomegaly</td>
<td></td>
</tr>
<tr>
<td><strong>Laboratory criteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Cytopenia affecting ≥2 of 3 lineages</td>
<td>Hemoglobin &lt;90 g/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neutrophil count &lt;1 x10^9/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Platelets &lt;100 x10^9/L</td>
</tr>
<tr>
<td>4.</td>
<td>Hypertriglyceridemia and/or</td>
<td>Triglycerides &gt;2 mmol/L (HLH-94) / &gt;3mmol/L (HLH-2004)</td>
</tr>
<tr>
<td></td>
<td>hypofibrinogenemia</td>
<td>Fibrinogen &lt;1.5 g/L</td>
</tr>
<tr>
<td><strong>Histopathologic criterion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Hemophagocytosis</td>
<td>In bone marrow, spleen or lymph nodes. No evidence of malignancy.</td>
</tr>
</tbody>
</table>

**Criteria added in 2004**

|                                    |                             |
| 6.               | Elevated ferritin              | Ferritin >500 µg/L          |
| 7.               | Elevated soluble CD25 (s-IL-2rec) | >2400 U/ml                |
| 8.               | Decreased or absent NK cell function | Local laboratory reference used |

* Patients with bi-allelic mutations in an FHL-causing gene and a clinical picture suggestive of HLH do not require fulfillment of all clinical criteria for diagnosis. The clinical diagnosis of FHL is further justified by a positive family history, and parental consanguinity is suggestive.
Treatment

The natural course of HLH is a rapid deterioration, and it has been reported that only 5% of the untreated patients survive more than a year, and that most children die within 2-3 months. For purely secondary forms, the chance of survival has been reported to be just over 50%. Therefore, and regardless of whether primary or secondary disease is suspected, HLH treatment needs to be started without delay. The aim of the therapy is threefold: immediate suppression of the severe hyperinflammation, eradication of the cells that trigger stimulation of hemophagocytosis, and – in primary HLH patients or in patients with relapsing disease – to permanently restore a functional immune system by HSCT.

Historical overview of treatment

Early attempts to control HLH included splenectomy, intravenous immunoglobulins, exchange transfusions, corticosteroids and cytotoxic agents such as vinblastine, but these induced no or only short amelioration. Ambruso et al in 1980 reported on prolonged disease remission with treatment by epipodophyllotoxin, and similar results were later confirmed by other reports, using epipodophyllotoxins in combination with corticosteroids and intrathecal methotrexate (MTX). In order to achieve permanent remission, HSCT was performed; the first successful bone marrow transplant in HLH was made in 1985 by Fischer et al, and in 1996, 5-year survival was 21% after various therapies including HSCT.

To date, there are two evaluated treatments for HLH: treatment by the HLH-94 protocol or ATG-based therapy. Furthermore, an international treatment trial (HLH-2004) is ongoing.

The HLH-94 treatment protocol

The HLH-94 treatment protocol was the result of a wide-reaching international collaboration. Its main constituent was the epipodophyllotoxine derivate etoposide. Etoposide is a widely used antitumor drug and a prototypical inducer of apoptosis. At the same time, it is an effective lipid radical scavenger and lipid antioxidant. Furthermore, etoposide-induced and Fas-triggered apoptosis are intact in HLH patients. In addition to etoposide, the protocol included dexamethasone and, in selected patients, intrathecal methotrexate. For an overview of the protocol, see Figure 5, and for information on drugs currently used for HLH, see Figure 6.

The initial therapy of the HLH-94 protocol included eight weeks of intensive treatment with the aim of inducing disease remission. It consisted of 150 mg/m² intravenous etoposide twice weekly during the first two weeks and then once weekly, in combination with dexamethasone. Dexamethasone is a corticosteroid with good transmission over the blood brain barrier, which was given in an initial dose of 10 mg/m² for two weeks, followed by successively lower doses.
for a total of eight weeks. Intrathecal MTX was recommended for patients with progressive neurological symptoms and/or persisting abnormal CSF findings. Patients with non-familial disease that resolved within the initial therapy then stop treatment after eight weeks. Continuation therapy consisted of pulses of dexamethasone in combination with etoposide and CSA. Cyclosporin A is an immunomodulatory drug that inhibits IL-2 and suppresses CTL proliferation. Continuation therapy was recommended for patients with familial, persisting or recurrent disease, and its aim was to keep the patient alive and free of reactivation until a HSCT could be performed. Allogeneic HSCT was recommended after initial therapy as soon as a suitable donor was found for patients with familial, persisting or recurrent disease. The choice of conditioning regimen and graft-versus-host disease (GvHD) prophylaxis was left to the treating center, but the suggested conditioning was a combination of busulfan, cyclophosphamide, VP-16, and, if the donor was unrelated, ATG. Suggested GvHD prophylaxis was intravenous methotrexate in combination with CSA. Supportive therapy consisted of treatment of infections and of prophylaxis with cotrimoxazole for Pneumocystis jirovecii in addition to an appropriate antimycotic treatment. In the case of an identified viral trigger, additional antiviral therapy was encouraged.

Figure 5. The HLH-94 treatment protocol.

The first evaluation of results of the HLH-94 treatment protocol was published in 2002, and showed a gratifying overall increase of survival, with a 3-year estimated probability of survival of 55±9% overall, and of 51±20% in patients with familial disease. 3-year survival after HSCT was 62±12%. The study included results from 113 patients included during the first 4 years of the trial and it was published prior to trial termination as a consequence of the unprecedented
survival results. Early relapses on therapy often seemed to occur after the first two weeks when dexamethasone was tapered, and this provided the rationale to move the start of CSA treatment to up-front in the HLH-2004 treatment trial.

HLH-94 was effective also for patients with sHLH: an early introduction of etoposide-based regimens was the only significant variable for survival in a Japanese study of patients with sHLH. The risk ratio for death was 14 times higher in 21 patients who did not receive etoposide at all or later than 4 weeks after diagnosis, as compared to the 26 patients who received early etoposide.179

In paper I we present a summary of the results of the HLH-94 treatment study almost 5 years after the trial termination in December 2003. To facilitate comparison, the inclusion criteria were the same as in the report published in 2002. In this study, 249 patients were eligible for evaluation and the median follow-up was 6 years. Altogether, 91% of patients had a follow-up of 5 years or more after therapy start, and the patient followed longest had survived 14 years after therapy start and 12 years after HSCT. The longer follow-up in this study enforces the assumption that patients surviving after successful HSCT or >1 year after therapy termination in patients who have not received transplants are permanently cured, and that late reactivations are rare.

The survival figures were similar to those of the previous publication; the 5-year cumulative probability of survival was 54±6% overall, and 50±13% in familial patients. No patient with a familial history survived without transplantation. Twenty-five patients had a major change of therapy during the trial, and these patients fared worse than the remaining patients. The 5-year survival after HSCT in the 124 patients who received transplants was 66±8%. Survival seemed to be better for patients who received transplants in disease remission (72±10%) as opposed to in a state of active disease (58±15%), although this difference was not statistically significant. Altogether, 49 patients were able to terminate all therapy without HSCT and remained free of disease reactivation for >1 year; these patients were assumed to have suffered from sHLH.

A large proportion of the deceased patients died within the two months of initial therapy. It would have been important to elucidate whether the deaths were caused by the disease itself or were caused by therapy toxicity. Reported causes of death in these patients were mainly multi-organ failure or septicemia, both which can result from either toxicity or HLH, making discrimination between HLH-related and toxicity-related deaths difficult. However, in 97% of the deaths within the first year in patients who did not receive transplants, the patient died with signs of active HLH. Sequelae in patients alive at the last follow-up were reported in 28%, and these late effects were mainly neurological (in 19%). One patient developed acute myeloid leukemia (AML) 6 months after therapy start. Overall, HLH-94 therapy was effective in inducing permanent disease remission or keeping the patient alive up until HSCT in 71% of the patients.
The HLH-2004 treatment protocol

HLH-2004 is the international treatment protocol that still is open for inclusion. Therapeutically, it is a slightly modified version of the HLH-94 protocol with start of CSA already up-front, and addition of corticosteroids to the intrathecal therapy.

Treatment by HLH-94 or HLH-2004 seems to be effective also for other genetic hemophagocytic syndromes. For these syndromes, no uniform induction therapy has been proposed. In paper III, we report treatment results in patients with GS2 (n=5), XLP1 (n=2) or CHS (n=2) treated by HLH-94 or HLH-2004 therapy. With a mean follow-up of 6.0 years in surviving patients, all but one were alive. Seven received transplants and were alive with a mean follow-up of 5.6 years after HSCT. One XLP patient achieved long-term remission without HSCT and was alive 3 year after treatment termination. Neurological sequelae were reported in all survivors except for the XLP patients. This study emphasizes that HLH-directed therapy can be used also for other genetic hemophagocytic syndromes and that treatment can be started at once a hemophagocytic syndrome has been diagnosed, and that exact diagnosis of genetic underlying defect can come later. For improved knowledge and adequate evaluation of therapy, we proposed to register these patients in the HLH-2004 treatment trial and in future treatment trials for HLH.

Side effects of HLH-94/HLH-2004 treatment

The therapy used in HLH-94 and HLH-2004 is potent and often causes side effects. Known side effects of etoposide are nausea, diarrhea, cytopenia and liver impairment. High exposure to etoposide also increases the risk of development of myelodysplastic syndrome and AML many years after treatment termination. Side effects of corticosteroids include hyperglycemia, weight gain, hypertension, personality changes, osteopenia, and osteonecrosis. Intrathecal MTX is associated with aseptic meningitis and encephalopathy. Cyclosporin A may cause hypertension, decreased kidney function, electrolyte disturbances, edema, and seizures. Furthermore, CSA is associated to the posterior reversible encephalopathy syndrome (PRES). Symptoms of PRES are those of an encephalopathy and may not be distinguished from those of CNS-HLH. Neuroradiological findings on MRI typically include vasogenic edema and bilateral, mainly posterior changes in the white matter, possibly aiding in discrimination between PRES and CNS-HLH. PRES has been reported in four American patients treated by the HLH-2004 protocol. However, PRES is reported also in septicemia, shock, malignancy and rheumatologic disease, making it difficult to pinpoint CSA therapy as the culprit in HLH patients.

Cytopenia was a commonly reported side-effect in patients in paper I, and a reason for delayed or reduced doses of etoposide. However, cytopenia may also be a result of HLH and it is a therefore a delicate decision for the clinician whether to reduce or maintain treatment intensity.
in these patients. Patients treated by HLH-94 therapy have been reported to have developed malignancy,\textsuperscript{119,182,183} and one patient in paper I developed an AML. He received in total eight weeks of HLH-94 therapy and was in complete HLH remission when the AML was diagnosed. He subsequently was transplanted and survived. Although it is likely that etoposide contributed to the malignancy development, reduced tumor surveillance by NK and CTL caused by HLH may also have played a part. It is still too early to tell the long-term risk of malignancy in HLH patients treated by HLH-94 or HLH-2004 therapy.

\textbf{ATG-based therapy}

In France, a different approach to therapy has been used, based on anti-thymocyte globulin (ATG). ATG was chosen for its lack of myelotoxicity and high toxicity for T cells. It was administered for five consecutive days together with methylprednisolone. Since ATG has poor penetration through the blood brain barrier, all patients received additional intrathecal methotrexate. Thereafter, maintenance therapy with CSA was given. HSCT was initially performed only if a genoidentical donor was found but, due to a large number of relapses on this regimen, HSCT was later performed in all patients in partial or complete remission and with an acceptable donor geno- or haploidentical donor.\textsuperscript{184,185}

In 2007, results of this single-center treatment trial were published.\textsuperscript{185} In 38 FHL patients treated by the ATG-based immunotherapy 1991-2005, the overall survival was 21/38 (55%). The regimen was very effective in inducing a complete or partial remission (in 45/46 courses), but a high proportion of relapses was reported although the time to HSCT was short, with a median of 6 weeks. The median duration of complete remission was 1.3 months. Eight patients died prior to HSCT, 30 received transplants, and 21 of these (70%) survived after transplantation. The treatment had only reversible immediate side effects, but was associated with reactivation of EBV and progressive lymphoproliferative disorder in patients with previous ATG administration or other previous immunotherapy. An advantage of this regimen would be that the long-term risk of malignancy development in survivors is reduced.

Several factors make a direct comparison between the ATG-based therapy and HLH-94 difficult. HLH-94 recruited patients from countries with highly varying medical resources and different disease panoramas. The definition of familial disease differed between the studies. In the French single-center study, 10 patients received ATG-based therapy as second-line therapy. Comparison between ATG-based therapy and the HLH-94 therapy can only be tentative, and the problems of comparison of the different strategies would best be addressed by a randomized treatment study.
Hematopoietic stem cell transplantation

A successful HSCT restores the immune defect permanently in patients with FHL. It is recommended following induction therapy in the HLH-94 and HLH-2004 protocols, as well as in the French ATG-based therapy regimen.

Several studies on results after HSCT in HLH patients have been published. Horne et al have reported on results of HSCT after HLH-94 therapy in 86 patients transplanted 1995-2000, with a median post-transplant follow-up of 4 years. Overall, the estimated 3-year probability of post-transplant survival was 64±10%. In 43 children with reported active disease after initial therapy, the odds ratio for survival after HSCT was 2.75 (1.26-5.99) as compared to 43 patients with inactive disease, indicating that patients with a good initial response also fared best post-transplant. Mortality was mainly transplant-related and typically occurred within 100 days post-HSCT.

The French group in 2006 reported results from their single-center experience of HSCT. Overall survival was 58% with a median follow-up of 6 years. Mixed chimerism was reported in half of the children who received transplants, but sustained remission was achieved with a mixed chimerism of >20%. An important complication was veno-occlusive disease.

To diminish transplant-related toxicity, reduced-intensity conditioning (RIC) has been proposed. To our knowledge, no center has randomized the conditioning regimen, something that would be important in order to learn more about the optimal regime. In a single-center comparison of myeloablative and reduced-intensity conditioning, RIC was associated with a high proportion of engraftment but also with an increased mixed donor/recipient chimerism. Survival was significantly better after RIC, and although these patients had a shorter follow-up it seems that a conditioning regimen with lower intensity may be a promising strategy for the future, perhaps especially beneficial for patients with preexisting organ toxicity.

Possible future therapies

There is as yet no established salvage therapy for poor responders in HLH. As relapse of HLH is a substantial clinical problem, this is an important dilemma that needs to be addressed in the future. Several monoclonal antibody-based therapies are under consideration (Figure 6). Importantly, a continued international treatment collaboration is required for recruiting enough patients for meaningful trials, in particular if comparisons of randomized treatment strategies are to be performed.

Although survival of children with HLH has improved, further improvements are needed, both in order to develop more effective treatment strategies and to reduce treatment-related toxicity.
To treat a young, febrile, cytopenic child with cytotoxic and immunosuppressive drugs may be contrary to the instinct of the treating physician. However, the collected experience of patients with HLH is unambiguous: the primary objective must be to break the vicious circle of hyperinflammation or the patient will die from septicemia or progressive disease with multisystem organ failure.

**Figure 6. The mode of action of immuno-chemotherapeutic agents used in HLH**, as well as for some agents that could be of interest in future treatment trials. Ag=antigen. Etoposide, VP-16, is an inhibitor of topoisomerase II, which binds to DNA and functions as DNA protective both during DNA replication and transcription. VP-16 stabilizes the complex formed by topoisomerase II and the DNA, thus forming stable (nonrepairable) protein-linked DNA double strand breaks. Other cells recognize such DNA damage and eliminate the injured cells by apoptosis.⁹¹ **Corticosteroids**, such as dexamethasone or methylprednisolone, are cytotoxic to lymphocytes, inhibit the expression of cytokines and chemokines,⁹² and also interfere with dendritic cell differentiation and production of the fas-ligand.⁹³ **Methotrexate, MTX**, is an antagonist of folic acid and inhibits DNA synthesis.⁴ **Cyclosporin A, CSA**, binds and inhibits cyclophilin D, which in turn inhibits calcineurin activity and suppresses lymphocyte replication.⁹³,⁹⁴ The production and synthesis of IL-2 is also inhibited.⁹⁵,⁹⁶ **Anti-thymocyte globulin**, ATG, is a heterologous polyclonal anti-serum obtained by injecting human lymphocytes in animals; most commonly horse or rabbit. Various ATG preparations exist, which differ in stimulating antigens (peripheral lymphocytes, thymocytes or even T cell lines).⁹⁶
In addition to accepted treatments, there is a search for new agents with a hopefully synergistic effect on the hyperinflammatory state in HLH. **Monoclonal antibodies** targeting specific antigens are constantly being developed. Some of these may provide future treatment options. For instance, alemtuzumab, anti-CD52 monoclonal antibody (Campath®), is directed towards CD52, a protein present on mature lymphocytes, monocytes and dendritic cells, but not on the stem cells from which these derive. Alemtuzumab is well tolerated, and currently undergoing an NIH randomized study for both front-line and salvage treatment in patients with aplastic anemia196 (Risitano, 2011). It is currently used as salvage therapy for HLH in certain centres.197 There are case reports of its use as first-line therapy in HLH.597 Daclizumab, anti-CD25 monoclonal antibody that binds to and inactivates the alpha-chain of the IL-2 receptor, thereby inhibiting the IL-2 dependent lymphocyte activation pathway, has also been used.198 TNF-α inhibitor, etanercept or infliximab, is currently used in MAS199, and the IL-1 receptor antagonist anakinra has also been used200. Other cytokine blocking agents have also been proposed, such as anti-IFN-γ.201 Rituximab, anti-CD20, (MabThera®) targets B cells, and is used in EBV- and XLP-associated HLH in order to reduce viral load by elimination of infected cells. Picture adapted from Risitano, 2011196.
CNS disease

One of the main complications of HLH is infiltration of activated lymphocytes and macrophages into the brain, i.e., CNS-disease. Furthermore, relapses of HLH during or after therapy often seem to occur in the CNS.

The frequency and character of acute clinical neurological symptoms have been described previously in this thesis, as well as the frequency of pathological neuroradiology and CSF findings. Although it is known that the CNS often is affected in HLH, no uniform definition of CNS disease has been presented. Haddad et al in 1997 reported that CNS disease, as defined by symptoms, CSF findings or neuroradiological pathology, was present in 85% of the patients at onset. In paper II, we found CNS disease in 63% of the patients. However, the definitions used for CNS disease were different, with Haddad et al including presence of neuroradiological pathology for CNS disease and used a higher cut-off for CSF cells (20 x10⁶/L). Nevertheless, we can conclude that CNS disease is common in the acute phase of HLH.

The three most commonly reported neurological findings in paper II were quite unspecific in nature: seizures, meningistic signs and irritability. Neurological evaluation of a severely ill and very young child may be difficult. However, we found that a pathological CSF was often also present in patients with these symptoms. Late sequelae in survivors were reported in 15% overall. Patients who had not received transplants and had a follow-up of >1 year after treatment termination without reactivation of symptoms, i.e., presumed secondary patients (n=37), had no neurological sequelae. Two non-transplanted patients, both still on therapy, had severe developmental delay upon follow-up. In the group of survivors after HSCT, 21% were reported to have neurological sequelae, most commonly neurodevelopmental retardation and epilepsy. To investigate whether CNS disease was a risk factor for outcome, patients were divided into groups related to the presence of neurological symptoms and CSF findings. We found that patients with neurological symptoms but a normal CSF exam had a sex- and age-adjusted HR for death of 0.98 (95% CI 0.42-2.31), that patients with pathological CSF but no neurological symptoms had a HR of 1.52 (0.82-2.82), and that patients with both CSF findings and neurological symptoms had a HR of 2.05 (1.13-3.72), all compared with patients with normal CSF and no neurological aberrations upon examination. Presence of pathological CSF was significantly associated with adverse poor probability of survival and increased probability of neurological sequelae, but neurological symptoms were not. However, patients with reported normal neurology but abnormal CSF examination were significantly younger than the remaining patients. Again, these findings possibly reflect the difficulty of neurological assessment in the very young child, and we conclude that CSF findings may be a more robust and therefore important measure in the evaluation of CNS disease.

It has not been clearly elucidated whether the intrathecal therapy of MTX used in both HLH-94/2004 and the ATG-based regimen is beneficial. With ATG-based treatment, intrathecal
therapy is used in all patients regardless of CNS disease. The rationale behind this is that ATG does not pass the blood brain barrier and methylprednisolon, the corticosteroid used, also has less penetrance. Treatment of CNS disease in the HLH-94 and HLH-2004 protocol consists of systemic etoposide and dexamethasone, that both have good penetrance over the blood brain barrier. Furthermore, intrathecal MTX is recommended for selected patients, and in HLH-2004 also intrathecal prednisolone. Evaluation of the efficacy of intrathecal therapy is difficult with this study design. In the report by Henter et al, 2002, 35 patients had CNS symptoms at diagnosis and the same percentage had normalization of symptoms with intrathecal therapy as without.119 These results could imply that intrathecal therapy was superfluous, but must be interpreted with care because of confounding by indication. In a French study by Haddad et al,146 neurological symptoms developed after start of therapy in all patients treated with chemotherapy/immune therapy only, and regardless of the number of intrathecal doses of MTX given.146 With ATG-based therapy,185 there was a significantly lower survival in patients with neurological disease at onset as opposed to those without. For firm conclusions to be drawn on the efficacy of intrathecal MTX a randomization would be necessary.

Risk estimation

HLH is a heterogeneous disease. Both patients with familial and secondary forms may have a variable disease severity, and both forms may be fatal early during initial therapy. It seems that FHL patients almost invariably require HSCT for permanent cure. Early pre-transplant fatalities still remain a therapeutic challenge and an early adjustment of treatment intensity depending on the risk of early death might be one tool to improve pre-transplant survival. In order to identify poor-responders at an early phase, we wanted to investigate risk factors for pre-transplant death.

Risk factors for poor outcome in HLH have not been elucidated. Outside the scope of a treatment study, risk factors are difficult to evaluate due to a lack of standardized treatment and evaluation. In a Japanese study of 82 patients with sHLH, values of ferritin, LDH and inflammatory cytokines had normalized in responders but not in non-responders, suggesting that these could be markers of therapy response.74 A recently published article reported that a rapid decline in ferritin values after start of therapy was associated to increased survival. Patients with an early ≥96% decrease of ferritin had a 17 times greater likelihood of survival as compared to patients with a decrease of less than 50%.202 Furthermore, and as stated above, our group has found that CNS disease, and especially the more specific finding of pathological CSF examination prior to therapy start, is a risk factor for both death and neurological sequelae (paper II). These findings are well in line with experiences from the French group of ATG-treated patients.185

In paper I, we reported that age <6 months and CNS disease at onset were associated to decreased survival. However, if patients with presumed sHLH were excluded from the analysis
no such association remained, indicating that the effect of sHLH for survival might have been confounded by age and CNS disease.

In paper IV and paper V, we aimed at conducting a more systematic investigation of associations between early clinical parameters and outcome four months after start of therapy in patients who had not received transplants. In paper IV, 232 patients reported from three European centers were included. We found that hyperbilirubinemia, hyperferritinemia and CSF pleocytosis at diagnosis, and thrombocytopenia and hyperferritinemia two weeks into therapy were associated early death. Thresholds of values were chosen with a data-driven approach, necessary in a setting with few patients and many missing values. Since CSF pleocytosis had a high proportion of missing values and the threshold associated to poor outcome was very high, relevant to only a few patients in a clinical setting, this risk factor should be interpreted with care.

In a further step, we investigated 297 patients from the HLH-2004 study (paper V). We wanted to combine the previously described risk factors into a prognostic score that could help in clinical risk assessment. Parameters chosen for inclusion in the prognostic “onset score” were hyperbilirubinemia and hyperferritinemia before therapy start, and in the “two week score”, also thrombocytopenia and hyperferritinemia at two weeks into therapy. To avoid bias by missing data, we practiced list-wise exclusion of patients with \( \geq 1 \) missing value of the parameters included in the score. Adjusted HR for pre-transplant death was 6.9 (95% CI of 2.0-23.4) for patients with an onset score of 2 as compared to an onset score of 0, and 11.2 (1.4-92.3) for patients with a two week score of 3, and 13.2 (1.2-146.8) for patients with a two week score of 4 as compared to patients with a two week score of 0.

In paper V we further wanted to validate the risk factors found in paper IV, and examine the generalizability of our results in an international context of HLH patients. We therefore performed similar calculations as in paper IV, and found that hyperbilirubinemia and CSF pleocytosis at onset and thrombocytopenia and hyperferritinemia two weeks into therapy remained significant also in this cohort. In additional analyses, we excluded the 70 patients that were included also in paper IV from analyses and found that CSF cells no longer remained statistically significant, but that the other three above-mentioned predictors did. The concluded results from papers IV and V therefore imply that these are risk indicators also in an international context of HLH patients.

We hope that these simple, easily available clinical parameters might be of help in risk assessment of patients with HLH, enabling more bespoke treatments in the future.
Follow-up of HLH patients

With the increasing numbers of survivors of HLH, a standardized and relevant follow-up is important. Regular follow-up after HSCT is standard in most HSCT centers, but what is included in this program may vary. Furthermore, there are no guidelines of follow-up for survivors of secondary HLH who have not required HSCT.

Naturally, one important part of follow-up aims at discovering signs of relapse. In patients who have received transplants, monitoring the degree of mixed chimerism is part of this. An important conclusion from paper I is that late relapses in successfully transplanted or non-transplanted patients in remission are rare. Relapses typically occur during induction or continuation therapy or within a year after therapy termination in patients who have not received transplants. In paper I the latest reported relapse in a patient who had not received a transplant was 22 months after treatment start and 10 months after treatment termination. For patients who have received transplants, relapses are frequent during the first hundred days post-transplant. In paper I only two deaths occurred >1 year post-transplant, the latest 20 months after HSCT. However, with the introduction of reduced intensity conditioning regimens which may result in an increased proportion of mixed chimerism, later relapses may become more frequent.

Furthermore, follow-up aims at finding, preventing and treating sequelae from the treatment and/or the disease itself. In paper I, sequelae such as nutritional problems and/or growth retardation, hypertension, impaired renal function, obstructive bronchiolitis and hearing impairment were reported in 16% of the patients upon follow-up. Neurological sequelae were reported in 19% of the patients. In paper II, we found that neurological sequelae were reported in 15% of HLH patients upon follow-up. Importantly, in some patients a neurological handicap was recognized first at the time of school start, when increasing demands on functioning was posed upon the child. This further emphasizes the importance of a long and thorough follow-up, not least of neuropsychiatric disabilities, so that social, pedagogical and/or pharmacological measures can be taken early.

In addition, the risk of development of secondary malignancies after etoposide-containing treatment is increased, as previously described. However, this risk may also be increased as a result of a reduced immunosurveillance by CTL and NK cells. Therefore, HLH patients need a follow-up reaching far into adult life.

For patients with GS2, XLP and CHS, follow-up after HSCT seems equally or maybe even more important. In paper III, we found a large proportion of GS2 patients with neurological manifestations of disease at onset and with sustained difficulties upon follow-up. Similar findings have been reported from France. In the five GS2 patients in paper III, CNS manifestations were present at onset or came with relapses during therapy, indicating that
neurological symptoms upon follow-up were sequelae and did not represent a neurodegeneration. Furthermore, no progression of CNS symptoms was found upon follow-up neither in these nor in the French patients.

Neurodegeneration in CHS, however, seems to be progressive and it is thought that it is independent of the immune defect in the hematopoietic cells and therefore not ameliorated by HSCT. In paper III, the CHS survivor was reported to have learning difficulties at 14 years of age, nine years after therapy start, in spite of absence of initial CNS disease. The cause of these difficulties remain unclear for this patient. A close neurological follow-up in CHS survivors thus seems essential.

In patients with XLP, no known manifestations outside the immune system are known. Interestingly, in paper III we report of a verified SAP-null XLP patient in long-term remission without HSCT, after HLH-94 initial and continuation therapy only. Follow-up after treatment termination was almost 3 years. Since no patient with XLP who has not received a transplant is known to have survived beyond 40 years of age, this patient needs close follow-up.

We are pleased to note that a new type of patients - long-term survivors after HLH - have come into existence. It is our obligation to closely follow possible late-effects in these children, adolescents and adults, and to take measures against possible complications of the disease or the treatment. A standardized hematological/immunological, neurological and quality-of-life follow-up is warranted.

**GENERAL DISCUSSION**

Papers I - III and V in this thesis include data from patients registered in the HLH-94 or HLH-2004 treatment trials, the two international treatment trials for patients with HLH so far. The protocols are used in countries all over the world. This wide-spread use is gratifying, especially since the incidence of FHL is likely to be higher in countries where consanguineous marriages are common and health resources may be limited. However, different distributions of sub-types of HLH patients as well as differences in health care methods and resources across countries in the study introduce possible bias, such as selection and information bias.

Paper I is a descriptive review of prospectively collected data on 249 patients treated within the HLH-94 treatment study protocol. This is the largest treatment study on HLH to date, and median follow-up of the patients included was 6.2 years. The main outcome studied was overall and sub-group survival. Furthermore, the report aimed to describe the initial clinical presentation and causes and timing for death. Since analytic reviews of HSCT and CNS-disease in the HLH-94 treatment study had previously been published, these subjects were only briefly covered.
The inclusion criteria were the same as the criteria of the previous publication from the same study, allowing for comparison. These criteria were strict, with only familial patients or patients fulfilling all HLH criteria included. This may have resulted in inclusion of patients with a more severe or advanced disease. Furthermore, patients with a major change of therapy were also included in the analysis, and as their survival seemed to be poorer, this may have shifted survival results of the study to the worse. However, patients who died prior to treatment start ("intent to treat") were excluded, with an opposite effect. We had a high follow-up rate with 91% of the patients having a follow-up of ≥5 years after start of therapy, or after HSCT in transplanted patients. Overall probability of survival was 54%, which was a great improvement as compared to previous reports. However, a troublesome high proportion of early deaths during the initial and continuation therapy remains, and post-transplant deaths also leave room for improvement in the future.

Comparison of survival between the two published treatments for HLH, the HLH-94 therapy and ATG-based therapy, is difficult. A randomization and maybe also a risk stratification of patients would be optimal for future studies, but it is difficult to reach statistical power, i.e., recruit enough patients for significant differences to be detected, when dealing with a rare disease. Therefore international cooperation trials are indispensable for firm conclusions to be drawn. Whether the ongoing trial with HLH-2004 therapy is beneficial and well tolerated will be important information in the planning of future strategies for HLH therapy.

**Paper II** is a descriptive review of the frequency and character of CNS disease in 193 patients registered in the HLH-94 database, including an analytic assessment of how initial CNS disease influences survival and neurological late effects. It is the largest report on CNS-HLH to date. Data were prospectively collected, and inclusion criteria were strict with all patients either fulfilling HLH criteria or having a familial disease. Patients with intent to treat were included. Forty-four patients who lacked information of CSF examination at onset were excluded, but did not significantly differ with regard to baseline information or outcome from the included patients.

The results of this study indicate how common CNS-HLH is, and stress the importance of a thorough evaluation of initial disease, as well as the importance of follow-up for survivors. CSF results seem to be more relevant for CNS involvement than neurological examination, especially in the young children, and should therefore be performed as part of the initial assessment in all HLH patients, if possible. Neuroradiological examination at diagnosis is now recommended in all HLH patients. CNS-HLH is less common in patients with sHLH. Prompt systemic treatment of HLH as well as an early HSCT in high-risk patients is important. Whether intrathecal therapy for CNS-HLH is beneficial, remains to be elucidated.
With the increased awareness of CNS-HLH, the follow-up form for neurological evaluation in HLH-2004 has been adjusted from that of HLH-94, to enable a more systematic clinical evaluation of HLH in the CNS at onset and upon follow-up.

In paper III, patients were recruited from both the HLH-94 and the HLH-2004 treatment protocol. This was a summary of patients with the inherited hemophagocytic syndromes GS2, XLP, and CHS treated by HLH therapy. Since there are no standardized treatment regimens for patients with these syndromes, we wanted to investigate whether these patients also could benefit from HLH therapy. Although these are only a few patients, we could conclude that the majority responded well to HLH therapy: seven of nine patients reached HSCT, and eight out of nine were long-term survivors. Neurological late-effects were common in GS2 patients.

There is currently no established salvage therapy for HLH. A step in the direction of development of a salvage therapy, is identification of the patients for whom it would be beneficial. In order to enable administration of salvage therapy to non-responders, these patients should be identified early, before hyperinflammation has caused an irreparable damage. Papers IV and V represent efforts to identify readily available risk factors for poor pre-transplant outcome. Inclusion criteria for the patients were the same in both papers, but patient cohorts were different. Missing data provided a great challenge in the evaluation of our data, reflected in the large confidence intervals of our results and jeopardizing internal validity. However, the parameters with strongest statistical significance in paper IV could be validated also in paper V, indicating that they are also externally valid in a larger perspective of HLH patients.

In paper V, we present an onset risk score and a two week risk score that may help in identification of high risk patients, i.e., patients with an increased risk of pre-transplant mortality. To note, these parameters and the risk scores do not discriminate between patients with sHLH and FHL. In paper I, we describe some possible clinical discriminators of sHLH with a favorable outcome. For the future, a different study design will have to be sought in the effort to discriminate between these two patient groups.
CONCLUSIONS
GENERAL CONCLUSIONS

The overall aim of the works compiled in this thesis was to improve survival and reduce morbidity in HLH patients. We conclude that:

- Survival has increased dramatically with the introduction of etoposide-containing regimens. The HLH-94 international treatment trial today represents a standard of care for HLH.

- About half of the patients with FHL are long term survivors. All patients with familial disease require transplant for a permanent cure. HLH-94 therapy can be effective in inducing sustained remission also in patients with secondary HLH.

- Two thirds of the transplanted patients survive. Transplant should preferably be performed in a non-active disease state, but active disease should not preclude transplant.

- Patients with secondary disease more commonly present at older age, are more often female and less often have CNS-disease or hepatomegaly.

- CNS disease at onset is common. Moreover, it is important to recognize, since it is a risk factor for neurological sequelae as well as for death.

- Fifteen percent of long-term survivors had neurological sequelae with a wide severity range. A close follow-up of HLH survivors is warranted.

- HLH in patients with XLP, CHS and GS2 may be successfully treated by HLH-94 or HLH-2004 therapy.

- Clinical risk factors for poor pre-transplant outcome include hyperbilirubinemia at onset of therapy and hyperferritinemia and thrombocytopenia at two weeks.

- By combining clinical prognostic risk factors into risk scores at onset and at two weeks, high risk patients may be identified.
FUTURE PERSPECTIVES

As a result of an international collaboration, much has been achieved in the understanding and treatment of HLH. That said, there is still much to learn and further work is warranted.

Aims for improved therapy of HLH include reduction of early pre-transplant deaths, including a salvage therapy for non-responders. Valid risk stratification tools could lead to a therapy adapted for high-risk patients, as well as a reduced treatment intensity for standard-risk patients, thereby reducing pre-transplant morbidity and mortality. Reduced-intensity conditioning may prove useful to improve post-HSCT outcome. A close and standardized follow-up of long-term HLH survivors is crucial, both in order to discover possible secondary malignancies and neurological late effects, and to learn more about quality of life after HLH therapy. Prevention of FHL by genetic counseling and/or by in vitro fertilization with pre-implantatory genetic diagnosis in affected families is also feasible. Whether patients with FHL and other genetic hemophagocytic syndromes such as GS2, XLP and CHS diagnosed by genetic methods should be transplanted prior to development of symptoms is another question that merits further consideration. Gene therapy has been used in other inherited immune defects, but may be associated with an increased risk for the development of malignancy. With improved methods, gene therapy may be a possible treatment also for FHL. Clinical findings in FHL patients have helped us understand mechanisms of the normal immune system. With the avalanche of new knowledge of NK and T cell function, as well as of mechanisms of apoptosis, more targeted treatment strategies for HLH patients will be developed.

However, the most important step in cure of HLH is early recognition and treatment start. Therefore, an important aim for the future includes an increased awareness of HLH among pediatricians likely to first encounter these patients, such as sub-specialists in hematology, infectious diseases, rheumatology, neonatology, gastroenterology and intensive care. Although there are new tools to assist in diagnosing HLH, such as molecular genetic investigations and/or cell functional assays, these may be time-consuming and must not delay treatment start in a patient with clinical picture clearly suggestive of the disease. Furthermore, although highly sophisticated diagnostic tools now are now available at laboratories in the industrialized world, most FHL patients are born in countries with limited medical care resources and where these diagnostic tools are not available. Routinely used laboratory diagnostic and prognostic criteria are therefore still of importance. The HLH-94 treatment has now been used in a wide range of countries around the world.

The spectrum of HLH has widened in the last decade. We now know that HLH can develop in a wide range of other inflammatory, malignant or infectious diseases, as well as in severely ill patients in intensive care units. Furthermore, patients with severe pandemic influenza have developed HLH that has been responsive to HLH therapy. Elucidation of the mechanisms behind HLH in septicemia and viral infection are important goals for the future.
To study rare diseases is a tricky business. Without an international collaboration and a generosity in sharing data and experiences, it is impossible. A world-wide cooperation enabling randomized treatment trials is important for the improved future of these patients.
SVENSK SAMMANFATTNING

Denna avhandling omfattar kliniska studier av hemofagocyterande lymfohistiocytos (HLH), sannolikt den vanligaste medfödda immunbristsjukdomen med dödlig utgång. Sjukdomen delas upp i familjär hemofagocyterande lymfohistiocytos, FHL, en recessivt ärligt sjukdom som obehandlad vanligtvis snabbt leder till döden, och i sekundär HLH, sHLH, som oftast utlöses i samband med en allvarlig infektion, malignitet eller av en reumatologisk bakomliggande sjukdom. Att särskilja grupperna kliniskt kan vara omöjligt vid diagnos. Barn med sjukdomen utvecklar symptom på en ohämmad inflammation, som feber och mjältförstoring. Sjukdomen orsakas av en defekt i immunsystemets förmåga att avdöda målceller, men även defekt nedreglering av det egna immunsvaret, och drabbade patienter har aktiverade vita blodkroppar spridda i olika organ, där de bl.a. åter upp kroppens egna blodceller, s.k. hemofagocytos. Tack vare ett internationellt samarbete har behandlingsstudier för barn med HLH kunnat upprättas. Ökad kunskap om klinik och cellfunktion hos barn med HLH har visat vägen för även prekliniska upptäckter och ökat förståelsen för hur vårt immunsystem normalt fungerar.

I delarbete I presenteras en sammanställning av resultaten från HLH-94, den första internationella behandlingsstudien för barn med HLH. Idag är 5-årsoverlevnaden 55%, och de patienter som genomgår benmärgstransplantation (BMT) har en överlevnad på 67%. Totalt överlever 73% fram till BMT, eller klarar sig den förutan. Kliniska undergrupper av de 249 patienter som inkluderades i studien presenteras med avseende på debutsymptomen.

I delarbete II beskrivs förekomst av och symptom på HLH i centrala nervsystemet (CNS) hos 193 patienter från HLH-94-studien. CNS-HLH förekommer hos 63% och är en riskfaktor för både sämre överlevnad och kvarstående neurologiska besvär, det senare förekommande hos 15% av de överlevande.


I delarbete IV undersöks i vilken utsträckning det finns tidiga kliniska riskfaktorer, relaterade till dödlig utgång före BMT. I delarbete V valideras de riskfaktorer som beskrevs i delarbete IV i en annan kohort av patienter, och en klinisk riskskattningskala föreslås, som framgent skulle kunna tjäna som hjälp för riskrutinerings och in behandlingsbeslut.

Sammanfattningsvis syftar denna avhandling till att öka den kliniska kunskapen om sjukdomen HLH. Även om överlevnaden förbättrats avlider fortfarande en stor andel barn med sjukdomen, och behandlingen i sig är intensiv och långt ifrån riskfri. Att minska den tidiga dödligheten, liksom att öka överlevnaden efter BMT är viktiga mål för framtiden. En nogsmal utvärdering av CNS-HLH, liksom uppföljning av överlevare med avseende på neurologiska symptom, är av stor vikt. HLH-94 kan användas som behandling även för barn med med HLH närbesläktade tillstånd. Kändedom om kliniska riskfaktorer kan ge vägledning för den behandlande läkaren, och på sikt leda till minskad morbiditet och mortalitet hos barn med HLH.
ACKNOWLEDGEMENTS

This work was performed at the Childhood Cancer Research Unit, Karolinska Institutet, and funded by grants from: the Märta och Gunnar V Philipson Foundation, Sällskapet Barnavård, the Swedish Childhood Cancer Foundation, the Swedish Research Council, the Cancer and Allergy Foundation of Sweden, the Swedish Cancer Foundation, Queen Silvia’s Jubilee fund, the Coordination Theme 1 (Health) of the European Community's FP7 (within the CureHLH Consortium, Grant Agreement no. HEALTH-F2-2008-201461), and by the Stockholm County Council (ALF).

I am indebted to a great number of people for having been able to write this thesis. Mentioned below are but a few.

From my research sphere:

Most importantly, I am grateful to the patients and their parents, for generously agreeing to participate in these studies

Jan-Inge, my supervisor: for your never-ending enthusiasm, energy, availability, helpfulness and for wise advices related to both research, clinical and private life. For listening to my ideas. Thank you also for introducing me to your “research baby”: HLH, and for trusting me to take care of it during my PhD.

AnnaCarin, my “elder sister in HLH”: thank you for your good judgment, analytical skills, for being a good listener and for friendship.

Elisabet Berglöf and Désirée Gavhed: for friendship and for excellent help, knowledge and for your good judgment. You always make me happy when I am at the research unit!

Erik Onelöv: for making me believe, if only for short moments, that statistics is straightforward

Thomas Silfverberg and Martina Löfstedt: for help with data collection

Eva Rudd and Kim Ericson: for introducing me to clinical genetics and laboratory work

Scott Montgomery: for input on epidemiological and statistical issues

Thank you also to all other group members and group associates, past and present, for collaboration and for fun times shared together.
All co-authors, for generous contributions to the present studies

The Histiocyte Society: for providing an international forum for further learning

The Doctoral school for clinicians in epidemiology: for providing me with a relevant education packed into an agreeable form

From my clinical sphere:

Bo Magnusson: for making room for research in the busy registrar schedule of Astrid Lindgrens Children’s hospital

My colleagues, past and present, for interest in my research, for support, discussions, and company during my years as registrar

All my smart, fun and lively friends!

I am extra grateful to Béatrice Skiöld: research support, colleague, neo-consultant, psychiatric consultant, and foremost a loyal and fabulous friend, and all in one person!

My family:

Mamma: for always being there when I need you, and always with a voice of common sense

My brothers and sister with families:

Petrus, Elisabeth, Erik and Svante. Magnus, Helena. Erica, Marit, Olle, Ida, Oliver and Carl. Extra big thanks, Svante, for the illustrations!

Grandpa Ivan: for showing me how research can be a source of joy and humor

Anna-Lisa, Agneta, Hans, Maria, Jöns, Thomas, Helena, Clara, Anna and Wilma: it is a joy to count you to my family as well!

Elis och Nina: för er förmåga att leva i nuet och för att ni fått mig ifrån datorn när jag bäst har behövt det. För att ni gör mig så glad!

Johan: for your calm, your computer support, for your down-to-earth wisdom, for your patience and for your love. For Elis and for Nina. This thesis, and - so much more importantly - my life, wouldn’t be the same without you.
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“Nature is nowhere accustomed more openly to display her secret mysteries than in cases where she shows tracings of her workings apart from the beaten paths; nor is there any better way to advance the proper practice of medicine than to give our minds to the discovery of the usual law of nature, by careful investigation of cases of rare forms of disease.”

/William Harvey, 1657