FRONT COVER: Big and small steps forwards

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While we try to teach our children all about life,
Our children teach us what life is all about.
— Angela Schwindt

To each parent who has experienced a child with HLH
ABSTRACT

Haemophagocytic lymphohistiocytosis (HLH) comprises primary (inherited) and secondary forms. The primary forms typically present in young children and carry a very high risk of mortality. The secondary forms, which are the result of different disorders, can present in all ages with greatly varying symptoms. The findings of HLH are related to an overly active but ineffective immune response, with accumulation of activated macrophages and lymphocytes, and a toxic state of hypercytokinemia. The typical symptoms of HLH are prolonged high fever, hepatosplenomegaly and cytopenia. HLH may also cause a meningoencephalitis and significant neurological late effects. A marker of HLH is impaired or absent function of natural killer (NK) cells and cytotoxic T cells. A major subgroup of primary HLH is Familial haemophagocytic lymphohistiocytosis (FHL). FHL is a rare autosomal disease and thus far three disease-causing genes have been identified: PRF1, UNC13D and STX11. Untreated FHL is invariably fatal, with a median survival of 1-2 months. The only curable method is currently HSCT. Prompted by earlier treatment failures, the Histiocyte Society initiated a prospective international multi-centre study (HLH-94) that combined two previously reported regimens: chemotherapy and immunotherapy, followed by HSCT in known familial and/or persistent or relapsing disease.

Aims: The aims of this thesis were to extend the clinical knowledge and diagnostics of HLH; to evaluate the outcome of the HLH-94 study; and ultimately to improve survival.

Results: The overall survival of the HLH-94 treatment far exceeded previous results. The estimated 3-year probability of survival was 55% (95% CI ± 9%). The HLH-94 initial and continuation therapy was successful in a total of 88/113 children (78%, 95% CI 69-85%), in that they were either admitted for HSCT (n=65) or still alive with at least one year follow-up since onset (n=23). The overall estimated 3-year probability of survival post HSCT was 64% (± 10%). The use of a matched unrelated donor (MUD) gave survival results comparable to those achieved when using a matched related donor (MRD), with a hazard ratio (HR) for mortality of 1.02 (CI=0.39-2.68) for MUD compared with MRD. The adjusted HR for mortality when using a haploidentical donor compared with an MRD was 3.31 (1.02-10.76), and the HR for mortality when using a mismatched unrelated donor (MMUD) compared with the use of an MRD was 3.01 (0.91-9.97). Persistent disease activity at two months after start of therapy appears to indicate a worse long-term prognosis. The increased risk of mortality post-HSCT for these patients remained statistically significant after adjustment for potential confounding factors (HR=2.75, 1.26-5.99, p=0.01). It is often difficult to distinguish at the onset of disease whether a patient has a primary or secondary HLH. This is a major clinical problem as it affects the decision whether an HSCT needs to be performed or not. Four subtypes of NK cell cytotoxicity deficiency have been described. The cytotoxic deficiency can be restored in all subtypes except type 3. To study association with clinical outcome, we thus pooled types 1, 2 and 4 together and defined them as being non-type 3. The estimated 3-year probability of survival was 46% for type 3 and 75% for non-type 3 (p=0.012). None of the 36 type 3 patients attained a sustained remission (zone year) after stopping therapy without receiving an HSCT, as compared with 13/29 non-type 3 patients (45%, 95% CI 28-62%). Finally, type 3 patients were associated with a statistically significantly increased risk of having active disease or not being alive two months after start of therapy, as indicated by an adjusted OR of 4.80 (CI 1.38-16.66). This indicates that NK cell sub-typing may provide a valuable tool for clinicians to determine whether or not an HLH patient requires transplantation. At diagnosis, a high proportion of children displayed neurological symptoms and/or pathological CSF (122/193, 63%) (neurological symptoms only: 72/193 (37%); pathological CSF only: 101/193 (52%)). An increased risk of mortality for patients with both neurological symptoms and abnormal CSF findings was shown when compared with patients with no neurological symptoms and normal CSF (adjusted HR 2.05, 1.13-3.72). A study of genotype-phenotype associations revealed that the frequency of gene mutations varies with ethnicity. The disease-causing mutations in FHL also display different phenotypes with regard to age at onset and pathological CSF at diagnosis.

Conclusions: In order to perform a meaningful clinical study of a rare disease, a collaborative international effort is required. The multi-centre study HLH-94 provides a successful example of this. Treatment according to the HLH-94 protocol has led to a dramatic increase in survival, and the work presented in this thesis will hopefully have a further positive impact on the outcome of children with HLH worldwide.

Keywords: Haemophagocytic lymphohistiocytosis, treatment, hematopoietic stem cell transplant, lymphocyte cytotoxicity, central nervous system, neurological symptoms, genotype-phenotype.
LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


* These authors contributed equally
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<tr>
<td>AML</td>
<td>Acute Myeloid Leukaemia</td>
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<tr>
<td>ATG</td>
<td>Antithymocyte Globulin</td>
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<td>CI</td>
<td>Confidence Interval</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CsA</td>
<td>Cyclosporine A</td>
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<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<td>CTL</td>
<td>Cytotoxic T Lymphocytes</td>
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<td>FHL</td>
<td>Familial Haemophagocytic Lymphohistiocytosis</td>
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<td>GVHD</td>
<td>Graft Versus Host Disease</td>
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<td>HAPLO</td>
<td>Familial Haploidentical Donor</td>
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<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<td>HLH</td>
<td>Haemophagocytic Lymphohistiocytosis</td>
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<td>HR</td>
<td>Hazard Ratio</td>
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<td>HSCT</td>
<td>Haematopoietic Stem Cell Transplantation</td>
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<td>EBV</td>
<td>Epstein-Barr Virus</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IT</td>
<td>Intrathecal</td>
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<tr>
<td>LAK</td>
<td>Lymphokine-Activated Killers</td>
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<tr>
<td>LCMV</td>
<td>Lymhocytic Choriomeningitic Virus</td>
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<tr>
<td>MCMV</td>
<td>Murine Cytomegalovirus</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility</td>
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<td>MMUD</td>
<td>Mismatched Unrelated Donor</td>
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<td>MRD</td>
<td>Matched Related Donor</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>MTX</td>
<td>Methotrexate</td>
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<tr>
<td>MUD</td>
<td>Matched Unrelated Donor</td>
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<tr>
<td>NK cell</td>
<td>Natural Killer cell</td>
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<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>RIC</td>
<td>Reduced Intensity Conditioning</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<tr>
<td>Tregs</td>
<td>Regulatory T cells</td>
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<tr>
<td>TRM</td>
<td>Transplant Related Mortality</td>
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<tr>
<td>XLP</td>
<td>X-linked Lymphoproliferative Syndrome</td>
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This thesis is a clinical epidemiological study of Haemophagocytic Lymphohistiocytosis (HLH). The inherited form of HLH is called Familial Haemophagocytic Lymphohistiocytosis (FHL). FHL is a rare disorder in which the homeostasis of the immune system is disturbed. FHL mainly affects young children and has a high mortality rate.

In order to explain this disease the thesis begins with a basic introduction to homeostasis in a healthy immune system, in cell death as well as its regulation by genetics. An introduction to HLH itself and the outcome for affected children follows. Next there is a presentation and discussion of the results of the research in this thesis, which focuses on the clinical characteristics that predict survival and therapeutic success in the context of an international therapeutic trial, and finally conclusions and speculation on future perspectives.

The five papers comprising this thesis are included in the final section.

STOCKHOLM, APRIL 2009

AnnaCarin Horne
GENERAL BACKGROUND

HOMEOSTASIS
Homeostasis is the maintenance of relatively stable internal physiological conditions under fluctuating environmental conditions.

The immune system and homeostasis
The immune system in humans is an incredibly complex system, essential for human survival. It strives to preserve homeostasis by defending our body against pathogens, tissue injury or malignant transformed cells whilst at the same time avoiding excessive damage to host cells and tissues. Once this task is accomplished the attack should rapidly cease and the defence forces withdraw. To avoid attacks on self-antigens the immune system has developed mechanisms of “tolerance” and complementing mechanisms to suppress stimulated immune cells (Danke, et al 2004, Verbsky and Grossman 2006).

The immune system can be divided into two components: innate and adaptive immunity.

Innate immunity
In the frontline of protection from a pathogen are the innate immune defences. Physiological and anatomical barriers constitute a part of the innate immune system. Furthermore, innate immune response involve the recruitment and activation of monocytes, macrophages, neutrophils, natural killer (NK) cells, cytokines and components of the complement system (Wyburn, et al 2005). In addition to pathogens, tissue damage or loss of “self” may also alert the immune system. The quick response of the innate immune system causes a state of inflammation in the local tissue.

Monocytes, macrophages and dendritic cells are all antigen presenting cells. Monocytes, the largest cells in the blood, are derived from myeloid progenitor cells and represent 10% of circulating blood leukocytes in humans (Geissmann, et al 2008). When monocytes leave the bloodstream and migrate into the tissue they acquire new functions and new receptors and they develop into either macrophages or dendritic cells. Both monocytes and macrophages possess the ability to phagocytose and initiate a strong local response to various forms of damage. Dendritic cells are essential for triggering and regulating the T cell responses of the adaptive immune system (Geissmann, et al 2008). Previously monocytes were considered as mere precursors but recently they were discovered to also operate functionally in the immune response (Geissmann, et al
Macrophages localised in the connective tissue are defined as histiocytes, however the term “histiocytes” is also used to describe an aberrant accumulation in any tissue of mononuclear phagocytes.

NK cells are a component of the innate immune system and provide a first line defence against viral infections and malignancies (Moretta, et al 2001). NK cells express receptors that bind to major histocompatibility class I (MHC class I) molecules. Simplified, NK cells use MHC class I as a marker of self and will recognize lack of MHC class I as a signal of missing self, facilitating NK cell killing of aberrant cells. The killing of the target cell is mediated through release of toxic granules containing proteases such as granzymes and the membrane disruptive protein perforin which induces a cell death pathway (Voskoboinik and Trapani 2006) (which will be described later). NK cells can also kill their targets independent of perforin by death receptor mediated apoptosis. NK cells can be activated by various cytokines. This activation results in increased number of cells and increased cytotoxic activity. NK cells are involved in the down regulation of the adaptive immunity (Jordan, et al 2004) and can function as a bridge between innate and adaptive immunity.

Cytokines are proteins that are produced and secreted by one cell and affect the function of other cells, or itself, by altering its behaviour or properties. Essentially all cells can produce and respond to a cytokine. An important task of the cytokines is to regulate the immune response. Cytokines can have enhancing and/or inhibiting effects, depending on the type of cell interaction and co-existing signalling molecules. In the literature, cytokines have been subdivided after their mode of action. Interleukin (IL)-1, IL-6, IL-12, TNF-α and IFN-γ are considered to be pro-inflammatory. Cytokines exert their effect by binding to specific cell surface receptors. Such binding usually initiates an intracellular cascade, which regulates the transcription of different genes.

The complement system consists of proteins that can be activated after encountering a pathogen. The activation of this system leads to; direct lysis of pathogens or infected cells, a dramatic enhancement of the phagocytic activity and production of cytokines that attract phagocytes and other leukocytes from the bloodstream and make them accumulate locally and there activate the effector cells of the innate immunity.

Adaptive immunity

The innate immune mechanisms can act rapidly, but are limited in terms of specificity. In contrast, adaptive immune responses are slow to start but eventually become both powerful and quick to recall. The adaptive immunity involves
recognition of specific antigens and confers both specificity and an immune memory. It is not inherited but evolves within a person's lifetime. The cellular constituents of adaptive immunity are B and T lymphocytes. These lymphocytes have the ability to recognize different antigens and both populations can generate highly diverse antigen receptor repertoires (immunoglobulins on B lymphocytes and T cell receptors on T lymphocytes). These hypervariable receptors are integral membrane proteins. They are encoded by genes assembled by a recombination of separate gene segments during the differentiation of the cells. This gives rise to the extraordinary diversity that can produce sufficient amount of specific receptors for all possible antigens encountered. When activated, B cells differentiate into plasma cells that can secrete antibodies. This differentiation and activation is in part regulated by T lymphocytes and cytokines. T cells are characterised by a T cell receptor and these lymphocytes are critical in the regulation of the immune responses. T cells are usually divided into two major categories; CD8+ cytotoxic T lymphocytes (CTL) and CD4+ T helper cells.

CTLs monitor all cells in the body and are ready to kill any cells that express foreign antigen fragments in connection with MHC class I. CTLs kill virally infected or transformed cells by a process similar to the NK cell mode of action, by the transfer of cytotoxic granulae (containing granzyme B) and perforin but also via another death pathway (Fas – Fas-receptor) (Dhein, et al 1995). CTLs are activated to kill effector cells by a specific binding to the target cell under the influence of cytokines.

Depending on the cytokine milieu in which the T helper cell is activated, it will mature into distinct subsets of helper cells. For example, T helper 1 subsets produce IFN-γ, “help” macrophages and are important in the immune defence against viral infections. Another subset recently discovered is the regulatory T cells (Tregs). These CD4+ Tregs have emerged as active regulators of immune responses in both humans and mice. Adaptive Tregs are generated in the periphery and can suppress activated T cells and play an important role in the termination of the immune response. Treg cells express high levels of perforin and the toxic granzyme B also found in NK cells and CTLs (Grossman, et al 2004).

The innate and adaptive immune systems collaborate to destroy invaders, reciprocally enhancing each other’s action (Medzhitov 2007). Through release of cytokines and presentations of peptides, cells of the innate immune system initiate and direct adaptive responses. Conversely, B cells secrete antibodies that activate complement and identify targets for phagocytosis or lysis by cells of the innate immune system. The maintenance of immune homeostasis demands a close control over the activation and termination of this intricate system.
Cell death and homeostasis

A balance between cell proliferation and cell death is essential for the maintenance of tissue homeostasis. There are a number of different possible fates for old, damaged or useless cells, including senescence (i.e. the cessation of cell proliferation with a permanent arrest of the cell cycle) and autophagic cell death (literally, “self-eating”, with lysosomal degradation of cellular components due to nutrient deprivation) (Vicencio, et al 2008). However, the most studied form of physiological cell death is apoptosis. Apoptosis occurs in all tissues and is a mean of regulating the number of cells in the body. The rate of apoptosis varies widely from tissue to tissue. It also provides a pathway for the rapid disposal of cells that are abnormal, misplaced, non-functional, or potentially dangerous to the organism (Jacobson, et al 1997). Apoptosis is invaluable to keep us alive and healthy. The cells of the immune system, for example, are regulated through apoptosis, which is of great importance in the elimination of immune effector cells as well as deletion of autoreactive B and T cells (Fadeel and Orrenius 2005).

Apoptosis differs substantially from necrosis (pathological cell death). It is a form of energy-dependent cell suicide where each cell expresses the components necessary for self-destruction. Whilst the process clearly does not provide any benefit for the cell itself, apoptosis is of wider benefit to the organism as a whole. Cell death due to apoptosis occurs strictly by means of a genetically “programmed cell death”. The morphological features of apoptosis are cytoplasmic and nuclear condensation; fragmentation of nuclei into membrane enclosed “apoptotic bodies”; and surface expression of opsonic receptors that allow neighbouring parenchymal cells to rapidly phagocytose and digest the corpse (Savill and Fadok 2000). In all cases this preserves an intact cell membrane. One of the hallmarks of apoptosis is that cells undergoing programmed death are normally phagocytosed by macrophages without activating an inflammatory or immune response, probably due to the fact that the cell membrane remains intact, thereby avoiding damage to the surrounding healthy tissue. In higher eukaryotes this programmed cell death can be triggered by “external” or “internal” factors. External triggering involves the ligation of dedicated death receptors to soluble or cell-associated ligands (Rathmell and Thompson 1999). As an example, cytotoxic effector cells of the immune system including NK cells and CTLs utilize several different pathways to induce apoptosis in affected or malignant target cells. Internal triggering occurs when cells respond to environmental stress (e.g. heat, x-rays or ultraviolet irradiation) by altering the function of mitochondria (Danial and Korsmeyer 2004) which regulates the start of programmed cell death.
If the balance of apoptosis is disturbed, tissue homeostasis is affected and human disease can arise, either from excessive or insufficient cell death (Hetts 1998).

**Genetics and homeostasis**
The development of a normal human being and the maintenance of the homeostasis in our bodies are ultimately regulated by genetically encoded proteins. This genetic information is made up from the chemical code within the DNA molecules located in our chromosomes. The code consists of the four nucleotides; adenine (A), cytosine (C), guanine (G) and thymine (T). Humans have 23 pairs of chromosomes (22 pairs of autosomes and 1 pair of sex chromosomes). Within each pair, one chromosome is inherited from the mother and the other from the father. Genes are complete, functional units of DNA that encode a protein. Each chromosome harbours many genes and altogether a normal human being has about 20,000 – 25,000 protein-encoding genes (Human Genome Project, 2004). Only about 1 – 1.5% of the human genome is considered to encode proteins whereas the rest is made up of non-coding DNA (Lander, et al 2001). Each gene has a “locus”, i.e. a specific chromosome location. The alternative forms of a gene are described as alleles. If a person has the same allele of a gene on both chromosomes within a pair, that person is said to be homozygous. If the two alleles differ, the person is said to be heterozygous. Genotype refers to the alleles of a gene that a person carries. Phenotype refers to the actual characteristics, and depends on the genotype as well as other factors such as the environment. Normally each cell in the human body contains the same genetic information. However, different tissues have their specific appearance and functions which also can be altered. These differences may be achieved by a selective activation of genes in each cell type at a certain time.

**Disease-causing mutations**
In a population there are genetic variances. A change in the genetic code can either represent a mutation or a polymorphism. Mutations are changes in the DNA sequence that may cause or contribute to a disease. One mutation can result in varying phenotypes, and one phenotype can be caused by mutations in different genes (genetic heterogeneity). A polymorphism is usually described as an allelic variant occurring at a frequency greater than 1/100 within a population. Polymorphisms may contribute to disease susceptibility in complex diseases.
HAEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS (HLH)

A disruption of the immune homeostasis has devastating consequences. If the complex machinery dedicated to terminate the human immune responses is not functioning there will be an accumulation of activated immune cells which pose a threat to healthy tissue. In combination with a trigger, such as an infection, these uncontrolled cells can produce a toxic state of hypercytokinemia which then results in a rare but life-threatening condition: haemophagocytic lymphohistiocytosis, or HLH (Henter, et al 1991c).

Classification of HLH

HLH is not a single disease but rather the final resulting symptoms of different disorders. These disorders are all associated with a disturbance of the monocyte/macrophage immune pathway which results in a pathological accumulation of “histiocytes”. This has given rise to the name haemophagocytic lymphohistiocytosis. The term Hemophagocytosis is defined as the presence of various blood-derived cells in macrophages. This phenomenon has previously been regarded as a hallmark of HLH, although it is neither mandatory nor specific to the condition (Henter, et al 1998).

HLH can be divided into primary and secondary forms (Henter, et al 1998, Janka, et al 1998). The primary forms usually, but not always, present in young children and always carry a very high risk of mortality. The secondary forms can present at all ages and the symptoms can vary greatly: from transient to fatal. Primary forms of HLH are inherited conditions. A major subdivision of primary HLH is the familial form, Familial Haemophagocytic Lymphohistiocytosis (FHL) (OMIM 267700). In addition to FHL, three genetic immune deficiencies are associated with HLH, but these also display other specific features: Chédiak-Higashi syndrome (OMIM 214500) (Stinchcombe, et al 2004), Griscelli syndrome type 2 (OMIM 607624) (Stinchcombe, et al 2004) and X-linked lymphoproliferative syndrome (XLP) (OMIM 308240) (Nichols, et al 2005).

Secondary forms of HLH are acquired disorders associated with infections, malignancies, physical stressors or inflammatory disorders such as systemic juvenile rheumatoid arthritis (Janka, et al 1998, Ramanan and Baildam 2002). Patients with a secondary form of HLH may have a genetic predisposition to develop the syndrome, but this remains as of today unknown. Among the infection-associated forms of HLH, the potentially aggressive Ebstein-Barrvirus (EBV)-induced disease is one well defined entity. EBV-HLH is most commonly found in East-Asia (Imashuku, et al 1999).

Most episodes of both primary and secondary HLH are triggered by an in-
Infection (Henter, et al 1993a, Janka 2007a). The most commonly known triggers are viruses of the herpes group, especially EBV and cytomegalovirus (CMV) (Janka 2007a). However, bacteria, protozoae and fungi can also trigger the disease (Fisman 2000, Janka, et al 1998). Notably, in both the primary and secondary forms of HLH, the triggering factor is often unknown.

Clinical manifestations and diagnosis of HLH

Both primary and secondary HLH initially display the same clinical manifestations. The major findings of HLH are prolonged high fever, hepatosplenomegaly and cytopenia. Other clinical symptoms are lymphadenopathy, jaundice, and a non-specific skin rash. Neurological symptoms such as irritability, seizures and ataxia are frequent already at onset of disease, but may also develop during its course. Abnormal laboratory findings include hypertriglyceridemia, hypofibrinogenemia, hyperferritinemia and coagulopathy. Cerebrospinal fluid (CSF) analyses can be normal or show a moderate pleocytosis and elevated protein count. Soluble interleukin-2 receptor (sCD25), a marker of generalized inflammation, is usually increased to very high levels in active HLH (Komp, et al 1989). Another typical finding is the impaired or absent function of NK cells and CTLs (Schneider, et al 2002b). A histological evaluation of bone marrow, spleen or lymph nodes may show hemophagocytosis. This is not necessarily demonstrable at onset, but more frequently found in advanced stages of the disease. A failure to find hemophagocytosis should therefore not preclude the diagnosis of HLH (Henter, et al 1991b).

Some patients with HLH have an atypical presentation with severe CNS symptoms as the only initial manifestation (Haddad, et al 1997, Henter and Elinder 1992). Neuroimaging findings are usually extensive but non-specific (Goo and Weon 2007). The picture can mimic chronic encephalitis, acute disseminated encephalomyelitis, or in the case of infants, non-accidental trauma (Fitzgerald and MacClain 2003, Weisfeld-Adams, et al 2009). Because isolated CNS symptoms may precede systemic HLH symptoms by several months, there is a substantial risk that the correct diagnosis will be delayed. For the clinician this is vital to bear in mind because the CNS symptoms may progress rapidly, leading to potentially severe late effects or early death (Haddad, et al 1997, paper IV).

The clinical manifestations of HLH can be tied directly to immune deregulation, with hyperinflammation, an overexpression of cytokines, and an accumulation of activated lymphocytes and histiocytes in internal organs (Henter, et al 1991c). The high levels of the proinflammatory cytokines IL-1, IL6 and TNF-α
will result in fever. Cytopenia is attributed to high levels of cytokines such as TNF-α and IFN-γ in combination with hemophagocytosis. The hypertriglyceridaemia is caused by a decrease in lipoprotein lipase activity: a direct result of high levels of TNF-α which is known to lower the activity of the lipase (Henter, et al 1991a). Activated macrophages secrete plasminogen activator which leads to high levels of plasmin, resulting in the cleavage of fibrinogen, which in turn causes hypofibrinogenaemia (Janka 2007b). Activated macrophages also secrete high amounts of ferritin (Allen, et al 2008). Increased levels of sCD25 are due to membrane turnover in activated lymphocytes (Komp, et al 1989). The hepatosplenomegaly and the CNS symptoms are in all likelihood due to the infiltration and accumulation of activated histiocytes and lymphocytes in these organs (Ost, et al 1998).

The diagnosis of HLH can be made either because the child has a family history or confirmed genetic defect, or because the child fulfils certain criteria based on the clinical manifestations. The first diagnostic criteria for HLH were presented by the HLH Study Group of the Histiocyte Society in 1991 (Henter, et al 1991b). These criteria were later revised by the same group in 2004 when ferritin, sCD25 and NK-cell activity were added (Henter, et al 2007). In the absence of a family history or a verified genetic mutation, five of eight of the HLH-2004 criteria are required for diagnosis. The diagnostic guidelines from 1991 and 2004 are presented in Table 1. The clinician should be aware that the diagnosis of HLH may be very difficult. Some patients fail to meet the diagnostic criteria until later in the course of the disease and others may show transient improvements. As prompt treatment can be life-saving, the diagnosis will sometimes have to be based on a strong clinical suspicion of the disease.
Table 1  The diagnostic guidelines for HLH in 1991 and 2004.

<table>
<thead>
<tr>
<th>Requirement for diagnosis of FHL</th>
<th>The 1991 diagnostic guidelines</th>
<th>The 2004 diagnostic guidelines</th>
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<tr>
<td></td>
<td>A positive family history of FHL</td>
<td>Either:</td>
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<td></td>
<td>All five diagnostic criteria fulfilled.</td>
<td>A molecular diagnosis consistent with HLH, or</td>
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<td></td>
<td></td>
<td>Five of the 8 diagnostic criteria below fulfilled.</td>
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<tr>
<td>Clinical criteria</td>
<td>Fever: duration &gt;7 days</td>
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<td></td>
<td>Splenomegaly &gt;3 cm below the costal margin</td>
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<td>Laboratory criteria</td>
<td>Cytopenias affecting two or more of three lineages in the peripheral blood with haemoglobin &lt;90 g/L, platelets &lt;100 × 10^9/L, neutrophils &lt;1.0 × 10^9/L</td>
<td>Hypertriglyceridaemia and/or hypofibrinogenæmia [fasting triglycerides ≥2.0 mmol/L (1991 criteria) and ≥3.0 mmol/L (2004 criteria), fibrinogen ≤1.5 g/L or ≤3SD]</td>
</tr>
<tr>
<td>Histopathologic criteria</td>
<td>Haemophagocytosis in bone marrow or spleen or lymph nodes. No evidence of malignancy.</td>
<td></td>
</tr>
<tr>
<td>New criteria</td>
<td>Low or absent NK cell activity</td>
<td>Ferritin &gt;500 microgram/L</td>
</tr>
<tr>
<td></td>
<td>Soluble CD25 (i.e. soluble IL-2 receptor)</td>
<td>&gt;2,400 U/ml</td>
</tr>
</tbody>
</table>

1991 diagnostic guidelines for HLH adapted from (Henter, et al 1991b)  
2004 diagnostic guidelines for HLH adapted from (Henter, et al 2007)

Epidemiology of FHL
The familial form of HLH, FHL, is inherited in an autosomal recessive manner, where the affected individuals are born to asymptomatic carriers. It is a rare disease, with an estimated incidence of 0.12 per 100,000 children per year, i.e. one child per 50,000 live-born (Henter, et al 1991d). This number is probably an underestimation since the incidence study was performed before the genetic defects were known about. The incidence is influenced by several genetic factors and is higher in areas of parental consanguinity. In Japan the majority of FHL cases are concentrated in a single area (Ishii, et al 1998). FHL was previously described to usually affect young children, with 70–80% having an onset of disease below the age of one (Arico, et al 1996, Janka 1983), but with advances in molecular biology and genetic diagnostics this proportion may decrease as additional patients are diagnosed with a less typical clinical picture. The children usually get severely ill in a sepsis-like manner and display the manifestations of HLH as described above. Without treatment the condition is fatal, with a median survival of one to two months after diagnosis (Janka 1983). Affected children die from multi-organ failure, secondary infections or CNS disease.
Pathophysiology of HLH
The manifestations of HLH are characterized by impaired or absent function of NK cells and CTLs (Schneider, et al 2002b) and very high levels of proinflammatory cytokines (Imashuku, et al 1998). The underlying pathophysiological mechanisms will be discussed below. An impaired or absent function of NK and CTLs leads firstly to a failure to clear infections. It also causes a defect control of the immune response by impairing the down regulation of the immune response (van Dommelen, et al 2006). Ultimately, a toxic state of hypercytokinemia and organ infiltration develops. See Figure 1.

NK cells and CTLs kill their targets cells through directed release of toxic granules containing perforin and granzyme B (Grossman, et al 2004). Both perforin and granzyme B (together with other regulatory proteins) are stored in cytolytic vesicles of these cytotoxic cells. Perforin is a membrane disruptive protein and granzyme B is a protease (Voskoboinik and Trapani 2006). Perforin is required for the delivery of granzyme B (in granules) to target cells which will mediate the triggering of these cell’s death by apoptosis. To initiate this “kiss of death” there needs to be a close physical contact between the cytotoxic cell and the target cell. The cytolytic vesicles of the cytotoxic cells have to move (traffic) to the contact site; dock and fuse with the plasma membrane, and release their content (Menasche, et al 2005). As described below, all known disease-causing mutations of primary HLH seem to impair this process.

The pathophysiology of secondary HLH is not yet revealed. These patients may also have low NK cell numbers. Patients with active disease often display decreased NK cell function, but this is usually restored when in remission. It is possible that the development of HLH manifestations in secondary forms are also associated with genetic mutations, although these are still unknown.

Studies have shown that the cells central to the immune pathology in HLH (activated monocytes, CTLs and NK cells) are most susceptible to cytotoxicity mediated by Treg cells, and that the killing of target cells by Treg cells is also perforin-dependent (Verbsky and Grossman 2006). However, the specific role of Treg cells in the pathophysiology of HLH has not been elucidated to date.

HLH and associated genetic mutations
The first genetic discovery in association with FHL was a linkage to 9q21.3-22 (Ohadi, et al 1999) but only a few patients have been reported with this linkage and the responsible gene is still unknown. Further, three loci have been mapped by homozygosity linkage to 10q21-22 (FHL2), 17q25.1 (FHL3) and 6q24 (FHL4). Three disease-distinct genes causing FHL2-4 have also been identified:
Figure 1  Schematic overview of the pathophysiology of primary HLH.

Adapted from Arceci, 2008
for FHL2 mutations in the perforin gene (PRF1) encoding the protein perforin (Stepp, et al 1999); for FHL3 UNC 13D encoding the protein munc 13-4 (Feldmann, et al 2003); and for FHL4 STX II encoding the protein syntaxin 11 (zur Stadt, et al 2005). There is ongoing research which aims to identify other causative genes for FHL. Other primary immune deficiencies that also are associated with HLH include Chédiak-Higashi syndrome associated with mutations in LYST, Griscelli syndrome type 2 with mutations in RAB27 A, and XLP with mutations in SAP (Menasche, et al 2005). See Table 2.

Table 2  Primary haemophagocytic diseases and associated disease-causing genes.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Chromosome location</th>
<th>Gene</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHL (locus FHL1)</td>
<td>9q21.3-q22</td>
<td>Unknown</td>
<td>-</td>
</tr>
<tr>
<td>FHL (locus FHL2)</td>
<td>10q21-22</td>
<td>PRF1</td>
<td>Perforin</td>
</tr>
<tr>
<td>FHL (locus FHL3)</td>
<td>17q25.1</td>
<td>UNC13D</td>
<td>Munc13-4</td>
</tr>
<tr>
<td>FHL (locus FHL4)</td>
<td>6q24</td>
<td>STX11</td>
<td>Syntaxin-11</td>
</tr>
<tr>
<td>Griscelli type II</td>
<td>15q15-21.1</td>
<td>RAB27A</td>
<td>Rab27a</td>
</tr>
<tr>
<td>Chédiak-Higashi</td>
<td>1q42.1-q42.2</td>
<td>LYST</td>
<td>Lysosomal trafficking regulator</td>
</tr>
<tr>
<td>XLP</td>
<td>Xq25</td>
<td>SHD1A/SAP</td>
<td>SLAM-associated protein</td>
</tr>
<tr>
<td></td>
<td>Xq25</td>
<td>XIAP</td>
<td>X-linked inhibitor-of-apoptosis</td>
</tr>
</tbody>
</table>

The seven genes described above are all well documented in terms of their ability to play an important role in the normal cytotoxicity of NK cells and CTLs (Menasche, et al 2005). The first five genes are all responsible for components of the perforin-dependent pathway that induces apoptosis in target cells. Their involvement can either be the production of perforin, vesicle priming, vesicle intracellular trafficking, granule exocytosis, or the delivery of proteolytic proteins required for apoptosis and a normal regulation of the immune response (Figure 2). Mutations at any of these steps cause inadequate apoptosis and may result in the clinical manifestations of HHLH as described above. In XLP, both SAP and XIAP deficiencies are also associated with defective NK cytotoxicity (Latour 2007).
Figure 2 Primary HLH: mechanisms of defective granule-dependent killing.

Schematic figure adapted with permission from Bengt Fadeel, Karolinska Institutet, Sweden
Animal models of HLH
Infection with lymphocytic choriomeningitic virus (LCMV) and murine cytomegalovirus (MCMV) in mice closely resembles CMV infections in humans. Infection of perforin knockout mice by LCMV gives a histological and immunological phenotype that resembles HLH. A study of perforin-deficient mice infected with LCMV showed that the main contributor to the HLH phenotype was found to be IFN-γ (Jordan, et al 2004). In another study, PRF1-deficient mice infected with MCMV also developed an HLH-like syndrome which was mainly caused by the dysregulated production of TNF-α (van Dommelen, et al 2006). This study also showed that perforin is important not only for the induction of apoptosis of the infected target cell, but also for the downregulation of the immune response after an infection has been cleared. In 2008, a Griscelli syndrome type 2 murine model of HLH was presented where Rab27a-deficient mice were infected with LCMV (Pachlopnik Schmid, et al 2008)). The first animal model of EBV-associated HLH was established by Hayashi et al (Hayashi, et al 2001). In this model, rabbits were inoculated by herpesvirus papio (HVP). All of the rabbits developed symptoms of HLH and died within one month. Although not entirely similar to the disease entity of human EBV-HLH, this animal model can serve as a valuable tool for investigating its pathophysiology (Chuang, et al 2007).

Treatment of HLH
Without therapy, familial HLH (FHL) is lethal. It is therefore vital that appropriate treatment is started promptly. Secondary HLH may resolve spontaneously but can also result in a life-threatening condition. The therapeutic strategy in secondary HLH is to eliminate the underlying condition, but there is often also a need to treat the syndrome specifically.

The first report of a successful treatment attempt of FHL with chemotherapy was published in 1980 (Ambruso, et al 1980). However, a thorough review in 1983 of all published cases thus far showed that with or without treatment, the median survival was less than two months, and one-year survival was close to 0% (Janka 1983). It was therefore obvious that treatment with chemotherapy alone was not a cure for the disease. In 1986 a treatment breakthrough was made when Fisher et al proved that FHL could be cured by allogeneic haematopoietic stem cell transplantation (HSCT) (Fischer, et al 1986).
The HLH-94 and HLH-2004 Treatment Protocols

In 1985, a small group of physicians interested in studying disorders related to histiocytes formed the international Histiocyte Society. In 1994 the FHL study group of the Histiocyte Society created a treatment protocol for HLH, called HLH-94. The aims of the study associated with the protocol were to control the life-threatening symptoms and achieve a state of disease remission so that the patients with FHL could be cured by an HSCT. The treatment consisted of a combination of chemotherapy (etoposide), corticosteroids and immunotherapy (cyclosporine (CsA)) as well as a focus on intensive supportive care. In earlier treatment recommendations, a firm FHL diagnosis was required before treatment was given. As the distinction between FHL and secondary HLH may not be possible in the initial clinical setting, this meant that many children died without treatment before diagnosis. A major advance in the HLH-94 protocol when compared with previous recommendations was that there was no requirement to separate FHL from the secondary forms, meaning that all children that fulfilled the diagnostic criteria for HLH were able to commence treatment. After eight weeks of initial treatment, patients with positive familial history of FHL and those with persistent or relapsing non-familial disease proceeded to continuation therapy and allogeneic HSCT. Children with a resolved non-familial, non-genetically verified disease after eight weeks of therapy discontinued therapy and restarted only in the event of reactivation. Those who did not relapse were taken off therapy and just monitored clinically (Figure 3) (Henter, et al 1997).

Figure 3: Flow-sheet for Children with Haemophagocytic Lymphohistiocytosis (HLH) in HLH-94

\[\text{All patients} \rightarrow \text{Register} \rightarrow \text{Initial 8 weeks chemotherapy} \rightarrow \begin{cases} \text{Familial disease} & \rightarrow \text{Continuation therapy and BMT if acceptable donor} \\ \text{Persistent non-familial} & \rightarrow \text{Continuation therapy and BMT if acceptable donor} \\ \text{Resolved non-familial} & \rightarrow \text{Stop therapy} \\ \text{Reactivation} & \rightarrow \text{Continuation therapy and BMT if acceptable donor} \end{cases} \]

1 Certain patients with secondary HLH may also need specific therapy.
As the only currently cure for FHL is HSCT, the study also had the aim to evaluate the results of HSCT with various types of donors. Since most affected children do not have any HLA-identical relative, the protocol suggests the use also of other donors, in particular matched unrelated donors, or if this is not possible, unmatched donors. In HLH-94, the suggested myeloablative conditioning comprised busulfan \( \text{po} 4\text{mg/kg body weight twice daily on days } -9 \text{ to } -6 \), etoposide \( 300\text{mg/m}^2 \text{ iv once daily on days } -5 \text{ to } -3 \), cyclophosphamide \( 50\text{mg/kg iv once daily on days } -5 \text{ to } -2 \), and antithymocyte globulin (ATG) as additional immunosuppression for unrelated donor transplants (Henter, et al 1997).

In 2004 a moderately revised update of the HLH-94 protocol was presented: HLH-2004 (Henter, et al 2007).
AIMS OF THE THESIS

The general aims of this thesis were to extend the clinical knowledge and diagnostics of HLH and to ultimately improve survival of affected children. The specific aims were as follows:

In **paper I**
- to analyze the survival of children with HLH after treatment with the HLH-94 protocol
- to analyze the efficacy of the initial and continuation therapy of HLH-94

In **paper II**
- to evaluate whether survival following HSCT is affected by the type of donor used
- to evaluate if disease activity at the time of HSCT affects survival
- to evaluate if disease activity after two months of induction therapy affects post-HSCT survival

In **paper III**
- to evaluate whether the type of NK cell cytotoxicity deficiency has any clinical significance

In **paper IV**
- to describe the frequency and nature of HLH-CNS manifestations at diagnosis as well as neurological late effects
- to study the relationship between the existence of neurological symptoms and/or pathological CSF at diagnosis and long-term outcome

In **paper V**
- to study the associations between genotype and phenotype in patients with FHL.
MATERIAL AND METHODS

STUDY POPULATION

Collection of clinical data
In papers I-IV patient data were extracted from the HLH-94 database in Stockholm. Comprehensive patient information was submitted to this database at regular intervals following start of HLH-94 treatment by treating physicians and national coordinators on follow-up forms. In paper V detailed clinical data were collected either by retrospectively reviewing patients’ records and/or by a questionnaire sent out to treating physicians. Information for paper V was collected on clinical and laboratory findings at onset of disease, treatment, response to treatment and long-term on outcome.

Inclusion of patients
The inclusion criteria for papers I, II and IV were: patients registered in the HLH-94 study who were aged 15 years or less at diagnosis, had no other disease and no previous cytotoxic or immunotherapy, and either all diagnostic criteria were fulfilled at diagnosis or familial disease (Table 1). In paper I 113 eligible patients started HLH 94 therapy between 1 July 1994 and 30 June 1998, were included in the study. The median follow-up time after start of therapy was 38 months for surviving patients (range 15–69 months). The patients studied were recruited from 21 countries. In paper II there were 87 transplanted patients who fulfilled the inclusion criteria and who had their first HSCT performed between 1 January 1995 and 31 December 2000. We report on 86 of these 87 children in respect of whom complete information existed on the covariates included in the multivariate analysis for evaluation on mortality following HSCT (the covariates are listed in ‘Statistical analysis’ below). The median follow-up time after transplantation in the 55 surviving patients was 4.1 years (range 1.1–7.2 years). In addition to the inclusion criteria described above, patients included in paper IV should have started treatment, or it had been intended that they would be treated, according to the HLH-94 protocol prior to 1 July 2003 (n=5). It was also required that complete information had been provided on CSF cell count and/or protein level at diagnosis, as well as a report on neurological findings at start of therapy. In total 193 patients were eligible for evaluation in paper IV. Of these, 102 patients had undergone HSCT, of whom 67 were alive at the time of analysis. The median follow-up time after HSCT with regard to evaluation of neurological late effects was 5.3 years (range 1.4–9.9 years).
In paper III a total of 68 patients diagnosed with HLH and registered within the HLH-94 database during the period July 1994 to June 2002 were evaluated for NK cell cytotoxicity deficiency subtypes. Each of the patients recruited was younger than 15 years old. Among these 68 patients, three patients were lost to follow-up, leaving 65 patients for the study. The median follow-up time following diagnosis (defined as onset of HLH therapy in the treated patients) was 4.6 years (range 0.6–7.8 years) in the surviving patients. The majority of the patients (n=58) were treated according to the HLH-94 protocol, five received no therapy or only corticosteroids, and two received other treatments.

A presentation of how many patients in papers I – IV were also included in the other papers is shown in Table 3.

Table 3  Number of patients included in multiple papers of this thesis

<table>
<thead>
<tr>
<th></th>
<th>Paper I (n=113)</th>
<th>Paper II (n=86)</th>
<th>Paper III (n=65)</th>
<th>Paper IV (n=193)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paper I (n=113)</td>
<td>-</td>
<td>63</td>
<td>30</td>
<td>91</td>
</tr>
<tr>
<td>Paper II (n=86)</td>
<td>63</td>
<td>-</td>
<td>22</td>
<td>75</td>
</tr>
<tr>
<td>Paper III (n=65)</td>
<td>30</td>
<td>22</td>
<td>-</td>
<td>37</td>
</tr>
<tr>
<td>Paper IV (n=193)</td>
<td>91</td>
<td>75</td>
<td>37</td>
<td>-</td>
</tr>
</tbody>
</table>

The study population in paper V was recruited via the Center of Molecular Medicine at Karolinska Institutet in Stockholm, Sweden. Between January 2000 and December 2006, DNA from 78 patients originating from the Nordic countries, Turkey and the Middle East was collected. Patients were initially included in the study if the treating physician had considered the disease to be, and treated it as FHL. One patient was later diagnosed with Griscelli syndrome type 2 and was therefore excluded from the genotype-phenotype analysis. In a second patient, sequencing of the PRF1 gene revealed a homozygous A91V gene alteration in the PRF1 gene, and since the pathogenic contribution of this mutation is unclear this patient could not be classified to a genetic subgroup. The patient was therefore excluded from the genotype-phenotype analysis and 76 patients remained in the study.

An overview of patient selection in the five papers of this thesis is presented in Table 4.
Table 4  Patient inclusion

<table>
<thead>
<tr>
<th>Paper</th>
<th>Number of patients</th>
<th>Study population recruited from</th>
<th>Recruitment period</th>
<th>Main outcome studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>113</td>
<td>HLH-94 study</td>
<td>Jul 1994 – Jun 1998</td>
<td>Overall survival and response to initial therapy</td>
</tr>
<tr>
<td>II</td>
<td>86</td>
<td>HLH-94 study</td>
<td>Jan 1995 – Dec 2000</td>
<td>Survival after HSCT</td>
</tr>
<tr>
<td>III</td>
<td>65</td>
<td>HLH-94 study</td>
<td>Jul 1994 – Jun 2002</td>
<td>NK cell cytotoxicity deficiency sub-types</td>
</tr>
<tr>
<td>IV</td>
<td>193</td>
<td>HLH-94 study</td>
<td>Jul 1994 – Jun 2003</td>
<td>Initial CNS findings and association with outcome</td>
</tr>
<tr>
<td>V</td>
<td>76</td>
<td>Center of Molecular Medicine, KI</td>
<td>Jan 2000 – Dec 2006</td>
<td>Genotype-phenotype associations</td>
</tr>
</tbody>
</table>

METHODS

Definition of HLH disease and therapy status

The disease HLH was defined by the diagnostic criteria established by the Histiocyte Society in 1991 (paper I, II and IV) or as defined by the treating physician (paper III and V). Non-active HLH disease was defined as having no clinical signs of disease, i.e. no fever except if infection-induced, no hepatosplenomegaly, no clinical signs of active CNS disease, and no cytopenias (except if drug induced), in accordance with the HLH-94 treatment protocol. CNS disease was defined as having an abnormal neurological examination and, in addition, CSF pleocytosis and/or elevated CSF protein. Pathological neurological findings were assessed by the treating physician at each referral centre (answering “yes” or “no”) and if neurological symptoms were confirmed, the clinician was required to specify these in free-text. The CSF was considered abnormal in the event of elevated leukocyte cell counts and/or protein levels (“yes” or “no” answers were reported, with values being provided in certain cases). Where the referring institution had provided a value but not confirmed abnormal or normal, we carried out this assessment using age-adjusted reference values (Behrman, et al 1996). To evaluate if neurological symptoms and/or abnormal CSF had any association with the long-term outcome (paper IV) we divided the patients into four CNS disease groups: normal CSF and no neurological symptoms (CNS group 1); normal CSF but neurological symptoms (CNS group 2); abnormal CSF but no neurologic symptoms (CNS group 3); and abnormal CSF with neurological symptoms (CNS group 4). With regard to their HLH
therapy status, the patients alive were classified as being “off-therapy” if they had been off therapy without disease re-activation for at least one year after stopping therapy and as “not off-therapy” if an SCT had been performed or HLH therapy had been administered during the last follow-up year.

Cytotoxicity assays (paper III)
The standard 51-chromium (Cr) release assay (4-hour) and 51-Cr release assay with modification by prolonging incubation time of effector and target cells to 16 hours have been described in detail previously (Schneider, et al 2002a). In brief, non-adherent lymphocytes were generated from peripheral blood of HLH patients. In the assays, lymphokine-activated killers (LAK) cells were generated by culturing peripheral blood mononuclear cells of patients in the presence of high-dose (103 IU/ml) recombinant human IL-2 for 72h. Phytohemagglutinin (PHA) was added to resident non-adherent lymphocytes to detect functional, most likely allo-restricted, cytotoxic T cells. In the 51-Cr release assay, un-stimulated and PHA activated peripheral blood lymphocytes as well as LAK cells were used as effector cells. The HLA class I and II negative K562 leukemic cell line was applied as a sensitive target cell throughout (Schneider, et al 2002b).

Classifications of cytotoxicity deficiency type (paper III)
Definitions of the cytotoxic deficiency types have previously been described in detail (Schneider, et al 2002a). In brief, type 1 NK cells lacked lytic activity against K562 cells in 4-hour 51-Cr release assay, cytolytic function was reconstituted in the presence of PHA but not by the rhIL-2 LAK protocol, and lysis at 16 hours was normal. The cytolytic function of type 2 NK cells with and without PHA stimulation in vitro mediated-lysis at 4 and 16 hours showed low values, but LAK cells generated in vitro showed normal lysis rates of K562 cells in 4- and 16-hour killing assays. Cellular cytotoxicity in type 3 NK cells was totally absent, and neither PHA or rhIL-2 stimulation nor prolongation of the incubation time of effector and target cells could restore the deficient cytolytic activity. Cytolytic activity of the lymphocytes of type 4 NK cells with and without stimulation of PHA and rhIL-2 was low or absent as determined in the 4-hour killing assay, but normal in the 16-hour assay. As described above, the NK cell cytotoxicity against K562 could be restored in all types except type 3. Hence, for analyzing association of NK cell cytotoxicity deficiency types with clinical outcomes, types 1, 2 and 4 were pooled together and defined as non-type 3 in the present study. See Table 5.
Table 5  Persistent NK cell cytotoxicity deficiency

<table>
<thead>
<tr>
<th>Subtypes</th>
<th>4h Resting</th>
<th>PHA</th>
<th>IL-2</th>
<th>16h Resting</th>
<th>PHA</th>
<th>IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Type 2</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Type 4</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Type 3</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

Adapted from Schneider et al 2002

Mutation detection of PRF1, UNC13D and STX11 (paper V)
Genomic DNA was isolated from peripheral blood or cultured fibroblasts according to standard procedures. Primers were designed for amplification and direct DNA sequencing of the coding region of the PRF1, UNC13D and the STX11 genes. The sequencing was performed on ABI 310, 3130, or ABI 3730 Genetic Analyzers (Applied Biosystems, Foster City, CA), and analyzed either using SeqScape (Applied Biosystems) or by hand. Seventy-two patients were analyzed for PRF1 gene mutations, 34 patients were analyzed for UNC13D gene mutations and 59 were analyzed for STX11 gene mutations. Unfortunately, all patients could not be analyzed for all three mutations due to lack of DNA. All families were sequenced at the Center of Molecular Medicine, Karolinska Institutet in Sweden, except three families from Oman analyzed at Sultan Qaboos University in Oman. For control samples in paper V, blood from healthy blood donors and healthy children at the Karolinska Hospital in Sweden was obtained after informed consent. A minimum of 50 healthy individuals, corresponding to 100 chromosomes, were analyzed for each detected mutation.

Statistical analysis
Differences in distribution were compared by using the Chi-square test, or where frequencies were small, the two-tailed Fisher’s exact test. Mann–Whitney U test was used to compare the difference in the median age at diagnosis (paper III and V). The survival rates were analyzed using the Kaplan–Meier life table method and univariate comparison of survival using the log rank test (paper I, II, III, and IV). Subsequently in paper II multivariate analysis using Cox proportional hazards regression was performed with time to death as the endpoint and using the maximum follow-up time available. The covariates used were sex, age at start of treatment, CNS involvement at start of therapy, disease activity at two months after start of treatment, disease activity at HSCT, time to HSCT, and donor type (matched related donors (MRD), matched unrelated donors.
(MUD), family haploidentical donors (HAPLO), and mismatched unrelated donors (MMUD)). In paper III multivariate analysis using logistic regression was performed with disease activity after induction therapy as the dependent variable. The covariates used were sex, consanguinity, age at start of therapy and NK sub-type group. In paper IV multivariate analysis using Cox proportional hazards regression was performed, with time to death as the endpoint and using the maximum follow-up time available. The covariates used were sex, age at start of treatment, if HSCT was performed, and CNS disease group. In paper V multivariate analysis using logistic regression was performed with age less than six months at diagnosis and pathological CSF as dependent variables. The covariates used were genetic mutation group and ethnicity. The SPSS 11.5 software (Chicago, IL) was used for all statistical analyses except the tests for associations between genotype and phenotype that were performed by exact Pearson chi-square tests for r×c tables using PROC FREQ in the SAS software. Differences were considered to be statistically significant where the p-value was less than 0.05. Odds ratio (OR) with 95% confidence interval was used to estimate the relative risk calculated by logistic regression. Hazard ratio (HR) was used to estimate risk calculated by Cox regression. Variables were included in the multivariate analyses if they were judged a priori to be associated with the outcome to improve precision or if they were assumed to be potential confounding factors.
RESULTS AND DISCUSSION

BACKGROUND
The HLH-94 study was the first international prospective multicentre study for patients with HLH. The hypothesis of the study was that the poor outcome for children with FHL (which, as explained above, is fatal without treatment) could be improved, as well as improving the outcome for children with secondary HLH. The treatment strategy of secondary HLH is firstly to treat the underlying disease. As there also is a risk of high mortality among secondary cases, this condition also requires specific therapy.

The HLH-94 treatment protocol is a combination of chemotherapy with etoposide/corticosteroids, and immunotherapy with CsA. In selected patients with CNS disease, IT MTX is also used. The major aim of the protocol was to initiate and continue a resolution of disease activity to a point in time when an HSCT could be performed. Earlier studies had showed that combining corticosteroids with etoposide (VP-16) was an effective treatment for prolonging survival in children with FHL. A treatment protocol including etoposide, steroids, IT MTX and cranial radiation was shown to be successful in inducing remission (Fischer, et al 1985). As well as a proposed treatment based on the similar drugs, but excluding cranial radiation (Henter, et al 1986). Immunotherapy with CsA had also shown to be effective. In HLH-94 these modalities were then combined (Figure 4).

The rationales behind the efficacy of the drugs in the HLH-94 protocol were based upon their pharmacological actions:

Corticosteroids (dexamethasone) have been used for more than 50 years and are well known for their anti-inflammatory effects. Corticosteroids will diffuse through the plasma membrane, interact with a specific steroid-receptor and enter the nucleus. The steroid/receptor complex can then regulate certain genes by modulating their transcription. Corticosteroids have wide ranging effects and affect many cells of the body. Of major importance for the hyperinflammation in HLH is the inhibition of the transcription factor NFkB, important for lymphocyte activation and cytokine production. This will lead to: Reduced production of proinflammatory cytokines (IL1, IL2, IL6, TNF, INF gamma, GM-CSF, decreased tissue accumulation of monocytes and macrophages) by inhibiting cell migration to the site of inflammation, inhibition of IL-2 and IL-2 signalling and promotion of the death by apoptosis in activated CD4+/CD8+ cells (Boumpas, et al 1993, Gerlag, et al 2004). Corticosteroids penetrate well.
Dexamethasone crosses the blood-brain barrier more effectively than prednisolone, and was therefore chosen to be included in HLH-94 treatment protocol.

Etoposide (VP-16) is a known excellent inducer of apoptosis, by stimulating the cells to express Fas and Fas-ligand on the cell surface. Etoposide mediates killing of pathogen-infected antigen-presenting cells and therefore reduces the stimulus for the ongoing but ineffective activation of cytotoxic cells (Ljungman and Hassan 2003). Etoposide also inhibits the synthesis of EBV nuclear antigen and has proven very effective in treating EBV-related HLH (Imashuku, et al 1999).

CsA is an immunosuppressive drug that is known to reduce T cell and macrophage activity. From the end of the 1970s it has shown to have a great impact on clinical transplantation. Immunosuppression by CsA is performed by disrupting the transcription of the IL-2 gene. Therefore, in the presence of CsA, IL-2 cannot be produced and the program of T cell activation, proliferation and differentiation is shut down at a very early stage. Furthermore, the activation of macrophages is limited (Parham 2005).

Methotrexate (MTX) is a cytotoxic agent important in the treatment of a number of hematologic malignancies. In addition to its anticancer modalities, MTX has also anti-inflammatory properties and is used in the treatment of inflammatory diseases (Ljungman and Hassan 2003). MTX can be administered intrathecally. In HLH-94 MTX is used only intrathecally, and in a limited number of patients, i.e. those with progressive CNS disease.
An established alternative treatment to HLH-94/HLH-2004 is immunotherapy based on ATG and CsA combined with corticosteroids and IT MTX injections (Mahlaoui, et al 2007). The possible mechanisms of ATG may involve elimination of activated CTLs or an alteration of the T cell function.

**THE FIRST FOUR PAPERS**

The four first papers of this thesis are based on patient data from the HLH-94 study, and form a part of the evaluation of the HLH-94 treatment protocol. The first paper (I) presents the outcome of children treated according to the HLH-94 protocol during the first four years of the study; the second paper (II) presents the outcome after HSCT; the third paper (III) describes how analysis of NK cell dysfunction can be of use in relation to the decision whether a transplant is needed or not; and the forth paper (IV) focuses on the associations between early CNS findings and long term outcome. As these papers all originate from the HLH-94 study, their results are presented and discussed together. The patients included in the separate papers are described above under “Methods”. One of the major selection criteria for inclusion (paper I, II and IV) was having either an affected sibling and/or fulfilling the 1991 Histiocyte Society diagnostic criteria (Henter, et al 1991b).

**Frequency of primary HLH in the study population**

When comparing different reports on HLH with regard to therapeutic results, it is important to be aware that the percentage of primary and secondary HLH per study may vary. In the inclusion period 1994–1998 of the HLH-94 study in paper I primary HLH could not be diagnosed by mutation analysis. The first genetic studies showing a linkage with a chromosome abnormality and FHL were not conducted until after the end of this inclusion period, in 1999. In papers I-III the disease was therefore considered to be familial if the patient had an affected sibling, either at the time of diagnosis or later. In both papers I and II, the percentage of children regarded as having familial disease was 22% (25/113 and 43/192, respectively). In paper II, 85/86 transplanted children were evaluated, and of those 29 (34%) had a known familial disease. By today’s methods of genetic testing, these proportions would be expected to be higher.

**Evaluation of overall survival**

The major aim of the HLH-94 study was to analyse survival and outcome after treatment with the HLH-94 protocol. In paper I we could show that the overall
survival of the HLH-94 protocol exceeded all expectations. At a median follow-up of 3.1 years, the estimated 3-year probability of survival of all 113 patients studied was 55% (95% CI ± 9%). Of the 25 children with familial history of the disease, the estimated 3-year probability of survival was 51% (± 20%). None of the familial patients survived without an HSCT. Twenty out of 25 patients with a known familial disease were transplanted, and of those 13 (65%) were long term survivors. These results confirm earlier findings that allogeneic HSCT can cure children with FHL (Baker, et al 1997, Blanche, et al 1991, Fischer, et al 1986, Jabado, et al 1997). The 3-year probability of survival is a reasonable estimate of the final cure rate, since very few deaths occur later than three years after onset of therapy. In paper I, 20 enrolled children were alive without disease and off therapy for more than 12 months without HSCT, presumably indicating that they had secondary forms of HLH. If these children were excluded, the estimated 3-year probability of survival was 45% (95% CI ± 10%).

Later studies have confirmed these promising results of overall survival: In paper IV the overall estimated 3-year probability of survival for the 193 patients included was 56% (95% CI ± 7%).

The overall probability of survival after treatment with HLH-94 exceeds treatment results presented prior to the study. The one year probability of survival as published by Janka in 1983 of 5% (5/101) can be compared with our crude survival rate one year after start of therapy, when 70/113 patients (62%) were alive (p=<0.001) (Janka 1983). One of the limitations of this comparison could arguably be that in 1983 no international criteria for HLH had been developed. The review by Janka was based on original patient reports of similar cases which had been described when published using various descriptive terms. In 1996 the FHL study group reported on 122 children with various treatments and an estimated 5-year probability of survival of 21% (Arico, et al 1996).

An alternative pre-HCST approach, based entirely on immune suppression including ATG, followed quickly by an HSCT (median six weeks) also showed a very high cure rate (Mahlaouï, et al 2007). This retrospective study based on the experience in a single institution between 1996 -2005, with data from 38 children, showed an overall survival in 21/38 children (55%, 95% CI 40–70%). Importantly in the absence of direct comparison between HLH-94 and ATG based therapy within the same study, conclusions regarding the effectiveness of the relative therapies can only be tentative. Further differences between the two studies are also worth noting: Mahlaouï et al’s conclusions were based on results from one highly experienced single centre while the overall survival of HLH-94 shown in paper I included patients recruited from 21 different coun-
tries. As HLH patients are critically ill, intensive supportive control has a great impact on outcome. With a multicenter study, it is natural that the modalities of intensive care can vary between countries. In paper I the largest single country including 31 patients displayed an estimated 3-year probability of survival of 71% (95% CI 53–84%). It is also worth noting that the quality of supportive care internationally may have improved over time. The inclusion period of paper I was 1994–1998 compared to the study of Mahlaoui et al which had an inclusion period from 1996–2005. Finally, a major difference between the two studies is the median time from start of therapy to HSCT – from six weeks in the single centre study of Mahlaoui et al to almost six months in the multicenter HLH-94 study.

Evaluation of initial therapy (week 1–8) and continuation therapy
Another aim of the HLH-94 study was to achieve disease resolution such that an HSCT could be performed, or in relation to secondary cases such that children were still alive and free of symptoms one year following start of therapy. In paper I we focused on the evaluation of initial and continuation therapy in the treatment protocol (it should be noted that according the HLH-94 protocol, initial therapy takes place until eight weeks after start of therapy, which in the papers of this thesis is referred to as being a period of two months). Initial and continuation therapy were successful in a total of 88/113 children (78%, 95% CI 69–85), in that they were either admitted for HSCT (n=65) or were still alive with at least one year follow-up since onset (n=23). Twenty of the 25 familial cases (80%) in the same study survived initial and continuation therapy, indicating a very high success rate in patients with FHL. In paper I the median follow-up time after onset of therapy in surviving patients was 38 months (range 15–69). The median time from onset of therapy to HSCT was six months, with the vast majority (86%) of the patients having been transplanted by 15 months. Therefore the study period was sufficient for most patients to have the possibility to be transplanted if needed. The Mahlaoui et al study showed that ATG-based immunotherapy of FHL can also provide sufficient resolution of disease activity, in that study allowing an HSCT to be performed in 30/38 of patients (79%, 95% CI 64–89%)(Mahlaoui, et al 2007).

Response to initial therapy
In the HLH-94 protocol inactive disease was defined as having no clinical signs of disease (no fever, no hepatosplenomegaly and no clinical signs of active CNS disease), and no cytopenia (except if drug-induced). In our study (paper I), in
response to initial therapy, 56/113 patients (53%, 95% CI 41–59%) achieved a resolution to inactive disease at two months, with partial remission in 34 cases (32%) and no response or death in 16 patients (15%).

This can be compared with the Mahlaoui et al study described above (Mahaoui, et al 2007). In their study of 38 patients they observed the results after 45 separate ATG courses with complete remission in 33/45 courses (73%, 95% CI 55–83%), partial remission in 11/45 (24%) of courses, with only one patient not responding at all. However, these data are not comparable as the time intervals differ: in our study the time of evaluation was two months after start of therapy. In the Mahlaoui et al study complete remission had a median time of eight days (range 4–15 days). In patients who did not receive a transplant shortly after ATG therapy, the median duration of complete remission was only 1.3 months with a considerable range (0.5–18 months). Further, the relapse rate after the ATG based therapy was higher than the relapse rate in HLH-94: 11/38 (29%, 95% CI 55–83%) vs. 7/56 (13%, 95% CI 55–83%). The incidences of relapse in the two studies cannot be compared since the time interval for relapse in the Mahlaoui study is not stated. The crude difference in relapse can partially be attributed to differential bias, but as these studies are not comparable no firm conclusions can be drawn.

The state of disease activity after induction therapy (active versus non-active) seems to affect long term outcome. In paper II (the HSCT study), 43/86 patients (50%) still had active disease after the two months of initial therapy. Our data indicated that children with active disease at two months had a significantly worse outcome after HSCT (51% ±15%) than those with inactive disease (77% ±12%) (p=0.009). This increased risk of mortality post-HSCT for these patients remained statistically significant after adjustment for potential confounding factors (OR=2.75, 1.26–5.99, p=0.011). This finding indicates that persistent disease activity at two months after start of therapy appears to indicate a worse long-term prognosis. This estimate may be a conservative measurement as we adjusted for factors that could have been a consequence of disease activity at two months, potentially resulting in over-adjustment.

In paper III we analyzed if there was an association between NK cell cytotoxicity deficiency subtype group and disease activity after two months. Patients who died of the disease or who were reported as having active disease were grouped in one group (active at two months) whereas patients reported with non–active disease were placed in the other group (non-active at two months). The NK cell deficiency subtype 3 was defined as cells where no reconstitution was seen, regardless of stimuli or prolongation time (as described in methods).
This subgroup was associated with a significantly increased risk of mortality or having active disease at two months as indicated by an adjusted odds ratio of 5.51 (95% CI 1.78-15.04). Adjustment for potential confounding factors altered these odds to 4.80 (95% CI 1.38-16.66). The biological differences associated with NK subtype group could perhaps be one of the explanations for our findings that patients with active disease at two months after start of therapy have a lower probability of survival overall and after HSCT (as can be seen in papers I and II). As only a limited number of patients included in the studies were investigated regarding NK subtype group, these associations could not be confirmed in these papers. In our papers patients with active disease at two months after start of therapy seem to have a worse prognosis. Disease activity at two months after start of therapy is a valuable measure in our international study since it is standardized for time and almost unaffected by HSCT. Active disease at this point in time may be a true indicator of a more aggressive disease, although it could also be an indicator of iatrogenic damage, or a marker of resistance to therapy. To conclude, it is possible that non-reversible NK cell cytotoxicity is associated with a disease that is more severe. It is also likely that disease severity is reflected not only in overall survival but also in the probability to achieve remission after two months of initial therapy. This is supported by the fact that as we, retrospectively reviewed the NK cell cytotoxicity subtypes of patients that later were found to have bi-allelic PRFI mutations, they all belonged to subtype 3.

**Side effects of initial and continuation therapy**

Both the chemotherapy and the immunotherapy used in HLH-94 are very potent and known to cause side effects. Side effects associated with dexamethasone include high blood pressure, increased appetite, weight gain, oedema, personality changes, muscle damage, softening of the bone, high blood sugar, pancreatitis and convulsions. Side effects associated with CsA include high blood pressure, decreased kidney function, headache, liver infection, electrolyte disturbances, edema and convulsions. CsA is also known to be associated with posterior reversible encephalopathy syndrome (PRES). In PRES there is a pattern of vasogenic brain oedema in the setting of neurotoxicity (Bartynski 2008). PRES consists of clinical symptoms of headache, confusion or decreased level of consciousness, visual changes and seizures associated with characteristic neuroradiological findings. Side effects associated with VP-16 include cytopenia, nausea, diarrhoea and transient liver function impairment. An increased risk of development of a secondary leukaemia also exists, with acute myeloid leukaemia (AML) and myelodysplastic syndromes having been reported as long-
term complications following the use of epipodophyllotoxin derivates (Henter, et al 1993b, Kitazawa, et al 2001). Side effects associated with IT MTX include neurological toxicity; aseptic meningitis, delayed leukoencephalopathy and acute encephalopathy. During the treatment with these medicines there is also a significantly increased susceptibility to infections.

In the HLH-94 study the existence of unacceptable side effects to therapy are reported with treating physicians being required to answer “yes” or “no” to the question whether or not they exist but specific report sheets for serious adverse events did not exist for this study. The major toxicity of the pre-HSCT therapy was neutropenia, in particular during the first two months of therapy, but since neutropenia is also commonly found in untreated HLH, it is sometimes difficult to determine to what extent it is due to therapy or active disease. Due to cytopenias, dose modifications were common during this period. In particular, the doses of VP-16 were decreased in a substantial number of patients. In paper I, 25/113 patients (22%) died prior to HSCT. Of these 25, 12 patients (48%, 95% CI 30–67%) were reported to have died during the first two months of therapy and 13 patients (52%, 95% CI 34–70%) died thereafter. The causes of death in 20/25 children (80%, 95% CI 61–91%) were considered by the reporting physicians to be due to progressive HLH disease. Four deaths were reported to be due to toxicity, and one after a diagnostic biopsy. Notably, it may sometimes be very difficult to clarify whether death was caused by the disease or by its treatment, in particular in cases of infections associated with neutropenia. In our total study population (paper I) one patient developed secondary AML which is most likely a complication following the use of VP-16. In paper I the median follow-up period for surviving patients was 3.1 years.

Evaluation of HSCT

An HSCT permanently replaces an individual's entire hematopoietic system, including the immune system. In the two to three weeks following a successful transplant, new circulating blood cells begin to be produced from the transplanted source, i.e. an engraftment has occurred. With a myeloablative conditioning before HSCT, the recipient's immune system and haematopoiesis are completely destroyed such that the patient would not survive without a transplant. The aim of the conditioning is threefold: to kill the dysfunctional hematopoietic or immune cells within the bone marrow, to provide room for the transplanted-functional cells, and to prevent rejection of the grafted cells by the recipient's T cells. With a myeloablative regimen, rejection of the new donor marrow is not a common problem. When an engraftment has occurred,
the mature donor CD4 and CD8 T cells accompanying the graft may attack the recipient's tissues. The condition caused by this effect is called Graft Versus Host Disease (GVHD) and is a major cause of morbidity and mortality after HSCT. The conditioning regimen damages not only the bone marrow but also other tissues typically those in which a rapid cell proliferation normally occurs, such as skin, intestines and the liver. These tissues are those where acute GVHD will be manifested. GVHD comprises acute and chronic forms. Acute GVHD develops within three months after transplantation. Chronic GVHD occurs by definition later than 100 days after transplantation. Whilst the chronic form is usually preceded by acute GHVD, it may occur de novo. To prevent GVHD, prophylaxis with immunosuppression is given to transplanted patients (Thomas, Textbook of Bone Marrow Transplantation).

In the HLH-94 protocol both the conditioning regimen and GVHD prophylaxis were decided by the treating transplant unit, although a suggestion was included in the protocol (Henter, et al 1997). The suggested myeloablative conditioning regimen consisted of Busulfan/ Cyclophosphamide/ VP-16, and for unrelated donors ATG. The suggested GVHD prophylaxis included short-course MTX in combination with CsA.

The compatibility between donor and recipient is determined by matching the human leukocyte antigen (HLA) system. HLA is synonymous with the human major histocompatibility complex (MHC). HLA are proteins in the cell membrane that function normally in antigen recognition of foreign agents and are important in the immunological recognition of foreign tissue. The MHC system is divided into two major classes: MHC class I proteins in humans are HLA-A, HLA-B, and HLA-C. MHC class II proteins in humans are HLA-DP, HLA-DQ, and HLA-DR. HLA-A, -B and-DR appear to be the most important loci determining whether or not transplanted cells will be rejected.

The methods to determine HLA types have become much more accurate during the past 15 years. The earliest HLA typing was performed by serological methods. Since the gene structure and sequence for the HLA molecules were detected and PCR technology was developed, a more specific molecular typing (genetic) was made possible. In paper II 18/86 donors (21%) were tested by serological methods and 50/86 (58%) by genetic methods. In 18 cases (21%) the method was not stated.

Donor selection has a great impact on outcome after HSCT. Matched related donors (MRD) are usually siblings. There is a 25% chance that a sibling will be HLA-matched. HLA-matched sibling donors are the preferred source for an HSCT in children with HLH. The risk of a sibling carrying the disease
must be considered and the donor should be tested for genetic markers and NK function assays. If an HLA identical sibling donor is not available, a search for a matched unrelated donor (MUD) is the next most suitable option. Today many national and international registries do exist which permit a coordinated worldwide search for an appropriate donor. However, it still can be difficult to find a matched unrelated donor, especially if the child in need of a bone marrow transplant comes from an ethnic minority. Alternatively mismatched unrelated donors (MMUD) can be used, who are then usually mismatched only at one major locus. If a related donor (most often a parent) is used that is only matched in one of the two HLA-haplotypes, i.e. a 1-3 antigen or allele mismatch this is called a haploidentical donor. Transplantations with haploidentical donors are only to be performed at experienced centres. The advantage of using a haploidentical donor is that within a given family, both parents (and siblings) may serve as potential donors and readily available donors. Today, in the absence of an HLA-identical sibling or unrelated donor, an unrelated cord blood transplant is often the preferred choice, rather than a mismatched related or unrelated donor (Gluckman, et al 2007).

In MRD, serological typing is essentially adequate since a match at these loci makes it highly likely that the same genes were inherited. In MUD a serological typing alone does not ensure that donor and recipient share the same HLA genes. Today HLA-typing in general is performed using genomic techniques for both Class I and Class II HLA-alleles, thus serological methods are not longer employed. Studies have shown that patients who are highly genetically matched with their donor have a better outcome (Speiser, et al 1996). Reducing the degree of possible mismatch with genomic typing on the allele level has improved the outcome of unrelated transplants.

As the only curative method of FHL currently is by means of an HSCT, a key aim of HLH-94 is therefore to get as many patients as possible in a stable state such that they can be transplanted, and quickly find a donor. Although outcomes following transplantation have improved, it should be borne in mind that HSCT remains a hazardous treatment and a transplantation should not therefore be performed unnecessarily, such as in milder secondary forms of HLH.

**Outcome after HSCT**

In **paper II** we analysed the outcome of HSCT among 86 children with HLH in more detail. These children all received HLH-94 therapy followed by allogeneic HSCT between 1995 and 2002. Information on engraftment was avail-
able for 83/86 patients. Of these 83 patients, 75 (90%, 95% CI 82-95%) were reported to have achieved engraftment. The three patients without information on engraftment all died during the first 100 days following HSCT. We identified an association between active disease at two months after start of therapy and primary graft failure. Of eight children who never engrafted, seven had active disease and one had inactive disease at two months (p=0.029). We could not correlate active disease at time of HSCT with engraftment (five had active and three inactive disease at transplantation). Due to the very small number of patients, no firm conclusions can be drawn from these data. These findings may, however, be of interest in that they are consistent with the previously described association of disease activity at two months and survival. Independent of clinical status at time of transplantation, patients with active disease at two months after start of therapy had decreased survival following HSCT. Among the 75 patients who engrafted, secondary graft failure was reported in three patients. These graft failures occurred within 100 days post HCST, and all three children died (one after a second transplant). In total, there were seven patients with recurrent HLH disease after HSCT; four of whom had no engraftment, two who had disease relapse despite sustained engraftment, and one with relapse without known information about graft failure. Six of these patients had their relapse in the early post HSCT period. One patient did suffer a late (450 days post HSCT) graft failure with relapse of HLH and subsequent secondary AML. HLH may relapse in the early post HSCT period but there seems to be a limited likelihood of late relapses. The pathophysiology of early HLH recurrence after transplantation in the presence of donor engraftment remains to be clarified. One possibility is that the engraftment recorded did not include NK cells and CTLs, and that the engraftment may therefore not have been complete regarding the cells important for HLH. Alternatively, the patients may have developed secondary forms of HLH.

Acute GVHD grades II–IV was reported in 25/78 patients (32%, 95% CI 23–43%). In another major study of HLH transplanted children where myeloablative conditioning was used, a similar proportion of reported acute GVHD grades II-IV were found: 36/88 (41%, 95% CI 31–51%)(Baker, et al 2008). In contrast, in another study where the same conditioning was used for the majority of children, acute GVHD grades II-IV was found in only 7/42 patients (17%, 95% CI 8–31%) (Ouachee-Chardin, et al 2006). In an attempt to minimise the incidence of GVHD and transplant-related mortality, trials with non-myeloablative conditioning (using reduced intensity conditioning (RIC)) have been performed (Cooper, et al 2006). In a single-centre study of 12 patients who received
the RIC regimen, a lower number of acute GVHD grades II-IV was found: 4/12 (17%, 95% CI 14–61%).

**Survival after HSCT**

In **paper I**, 65/113 children (58%) underwent an HSCT, with an estimated 3-year probability of disease-free survival after HSCT being 62% (± 12%). This outcome must be considered to be acceptable, not least in view of the fact that it was achieved with only a minority of the 65 transplantations involving an MRD (n=15). Previously, patients without an MRD would commonly not have been transplanted and therefore died.

The overall estimated 3-year probability of survival post HSCT in **paper II** was almost the same at 64% (± 10%). At the time of analysis, 55 children were alive (64%), with a median follow-up period of 4.1 years post-HSCT (range 1.1–7.2 years), and they were all reported as being free of disease. This probability of surviving three years must also be viewed in the light of the mixed clinical and geographical characteristics of the patients. The overall estimated 1-year probability of survival post HSCT in **paper II** was 66% (95% CI 56–75). Other studies of HSCT for patients with HLH have shown an estimated 1-year probability of survival to be 52% (95% CI 41–62) (Baker, et al 2008), and 60% (95% CI 46–73%) (Ouachee-Chardin, et al 2006). In a smaller study of 12 patients with RIC conditioning, 8/11 patients (73%, 95% CI 43–90%) were alive one year after HSCT and the authors recommend this treatment for patients receiving grafts from non-related donors and in patients with severe organ toxicity (Cooper, et al 2006), but more patients are needed to confirm these promising results.

In general, survival following HSCT for patients with HLH is lower than survival in patients with other non-malignant diseases. The survival post-HSCT in children with sickle cell anaemia has been reported as 78% 2-year estimated event-free survival (Locatelli, et al 2003) and, in another study, 55/59 children (93%, 95% CI 83–97%) survived more than one year (Walters, et al 2000). It is possible that the decreased survival post-HSCT in patients with HLH is associated with the fact that FHL is a disease affecting the down-regulation of the immune system itself.

**Mortality after HSCT**

It must be observed that in **paper I** 25/65 (38%) transplanted children had died, indicating that a major risk of fatality for HLH patients still occurred after HSCT. At the time of analysis in **paper II**, 31 of the 86 transplanted children (36%) had died. Of these 31 deaths, 26 (83%, 95% CI 67–93%) were reported to
be transplant related mortality (TRM), while two died after relapse of HLH, one died of secondary AML, one died of respiratory disease of an unknown cause, and one died during a surgical procedure unrelated to HLH. A total of 23 deaths (74%) occurred within 100 days after HSCT. The vast majority of deaths (94%) occurred within the first year after HSCT, only two deaths occurred during the second year, and then the survival curve was flat.

As described above the vast majority of the deaths were accounted for by TRM. The TRM in paper II was reported in 26/86 (30%, 95% CI 21–41%). Of the early deaths (< 100 days) following HSCT at least half of them were due to treatment complications related to the liver and lungs (veno occlusive disease and non-infectious pneumonitis). Similar findings have been observed in other series of HSCT in HLH were myeloablative conditioning have been used (Baker, et al 2008, Cesaro, et al 2008, Ouachee-Chardin, et al 2006). The TRM is quite high for patients with HLH compared with TRM in other non malignant diseases.

Impact of donor on outcome
Since most affected children with HLH do not have an HLA-identical relative, alternative donors have been increasingly used since the late eighties, in particular MUD but also mismatched donors and over the last 10 years also unrelated cord blood units. One of the general aims of the HLH-94 study was to evaluate the results of HSCT with various types of donor.

In paper II, of the 86 evaluated transplantations, MRD were utilized in 24; MUD in 33; haploidentical in 16 and MMUD in 13. For the individual donor groups, the estimated probability of survival three years after HSCT was; 71% (± 18%) for MRD, 70% (± 16%) for MUD, 50% (± 24%) for HAPLO and 54% (± 27%) for MMUD. We could demonstrate that the use of MUD provides survival results comparable to those achieved with MRD, as the hazard ratio (HR) for mortality is 1.02 (CI=0.39–2.68) for MUD compared with MRD.

Of the transplanted patients in paper II where HLA non-identical donors were used (n=29), 15 (52%) were alive at last follow-up, with a median follow-up of 50 months. With regard to unadjusted hazard ratio (HR) for mortality, there is no statistically significant difference between HAPLO and MMUD. However, the adjusted HR for mortality for HAPLO compared with MRD is 3.31(1.02–10.76), and the HR for mortality for MMUD compared with MRD is 3.01 (0.91–9.97). This may be due to effect modification by other disease characteristics, such as disease activity. However, the relationship is complex and could not be fully explained by effect modification by disease activity solely at
two months or solely at transplantation: as the number of patients is small, this may also represent a chance finding. Although the use of HAPLO and MMUD gives a less favourable outcome than the use of MRD or MUD, the outcome is still acceptable, supporting the use of alternative donors where matched donors are unavailable. We suggested in paper II that when transplantations involving HLA non-identical donors are considered, these should be performed in experienced centres.

Impact of disease activity at time of transplantation on outcome
Opinions vary as to what degree of disease activity immediately prior to transplantation compromises long-term outcome. In paper II we wanted to analyze if disease activity at time of HSCT affects survival. In this study of 86 children, there was a tendency towards better survival in patients with inactive disease at HSCT, but this failed to achieve statistical significance in univariate analysis (OR for mortality 1.93, 95% CI, 0.95–3.91). The increased risk of mortality post-HSCT for patients with active disease at two months after start of therapy remained statistically significant after adjustment for potential confounding factors (OR=2.75, 95% CI 1.26–5.99, p=0.011). This finding indicates that persistent disease activity at two months after start of therapy appears to indicate a worse long-term prognosis. This estimate may be a conservative measurement as we adjusted for factors that could have been a consequence of disease activity at two months, potentially resulting in over-adjustment.

In 2006 a report on the impact of disease activity at time of HSCT from a single-centre study including 48 children with HLH was published (Ouachee-Chardin, et al 2006). Children with active disease at time of HSCT seemed to fare worse (p=0.053). However, in children with matched-donors disease activity did not affect outcome significantly whereas it did in those transplanted with a haploidentical donor. Analysis including adjustment for donor types was not performed. A separate study of 98 children with unrelated donors (54 matched) was published in 2008 (Baker, et al 2008). This study showed an estimated 5-year overall survival after HSCT of 52%. Disease specific criteria were only available for 51 patients (56%). Forty-six of those 51 patients (90%) were in clinical remission at time of HSCT, with an estimated 5-year probability of survival of 49% (95% CI 33–62%). Only one out of five patients with active disease at transplantation was alive at last follow-up. The authors conclude that there was a higher mortality in patients where HLA non-identical donors were used but analysis including adjustment for donor types was not performed. In a study of 72 transplanted patients it was shown that disease activity at time of
transplantation was the only factor which significantly predicted outcome after HSCT involving a partly miss-matched related donor (Henslee-Downey, et al 1997).

**NK cell cytotoxicity deficiency subtypes**

As previously described, it is often difficult to distinguish at the onset of disease whether a patient has a primary or secondary HLH, especially if there is an absence of familial history of the disease or if disease-causing mutations have not been confirmed. This is a major clinical problem in relation to HLH as it also affects the decision whether an HSCT needs to be performed or not. Laboratory markers facilitating this therapeutic decision are very much needed.

Defective NK cell mediated cytotoxicity is a diagnostic criterion for HLH. As earlier described, NK cells and cytotoxic T cells have an ability to directly kill targets cells. Both types of cells require direct contact with target cells to kill, which can involve either of two pathways. The first pathway is non-secretory, and depends on the interaction between target cell death receptors and the ligands of these receptors on the killer-cell membrane. The second pathway involves perforin and cytolytic granzymes, which are secreted by exocytosis and cooperate in inducing apoptosis in the target cell (Shereck, et al 2007). It is suggested that NK cells distinguish normal, healthy cells from infected or damaged target cells by balancing signals from numerous activating and inhibitory receptors. NK activating receptors can be up-regulated by various means, for example by IL-2 stimulation. Activation of NK cells can increase their number and cytotoxic activity (Shereck, et al 2007). It is also shown that a mitogen (PHA) can stimulate NK cell activity (Schneider, et al 2002b). It has been proposed that patients with HLH may present with four distinct types of defects in their cellular cytotoxicity: 1, 2, 3 and 4, depending upon their ability to reconstitute NK cell cytotoxicity. Type 1 cells regained their capacity to lyse cells after agglutination with PHA. Type 2 cells regained function after stimulation with IL-2. Type 4 cells were able to regain function after prolonged stimulation. In contrast, in type 3 cells no reconstitution was seen, regardless of stimuli or prolongation time (Table 5).

In paper III we sought to uncover the potential clinical significance of grouping NK cells into different types, by studying the association of NK cell cytotoxicity deficiency types with clinical outcome (n=65). As described above, the NK cell cytotoxicity could be restored in all subtypes except type 3. We therefore pooled types 1, 2 and 4 together and defined them as being non-type 3.

All results in paper III indicate greater disease activity and less favourable
outcomes in patients with type 3 as compared with those with non-type 3: Type 3 patients had a lower probability of survival. The estimated 3-year probability of survival was 46% for type 3 patients and 75% for non-type 3 patients (p=0.012). None of the 36 type 3 patients attained a sustained (> one year) remission after stopping therapy without receiving an HSCT, as compared with 13/29 non-type 3 patients (45%, 95% CI 28–62%). Finally, type 3 patients were associated with a statistically significantly increased risk of having active disease or not being alive at two months after start of therapy, as indicated by an unadjusted OR of 5.51 (CI 1.78–15.04). After adjustment for potential confounding factors these odds remained significantly increased (4.80, CI 1.38–16.66). These results indicate that NK cell sub-typing may provide a valuable tool for clinicians to determine whether or not an HLH patient requires transplantation.

At birth NK cells are the major perforin-expressing lymphocyte subsets. NK cells are therefore important effectors of cytotoxic responses in infants, whose perforin-expressing T cells are still underdeveloped. In paper III we could also show that the frequency of patients who were six months old or younger at diagnosis was significantly increased in the type 3 group (21/36, 58%) compared with the non-type 3 group (7/29, 24%) (p= 0.006). This finding may indicate that decreased NK cell cytotoxicity in infants with FHL is less likely to be reconstituted, possibly suggesting that these individuals have more severe disease biology.

As of today, functional studies of NK cytotoxicity remain a valuable tool in the diagnostic evaluation of patients with HLH. Our laboratory at KI performs analyses after stimulation with IL-2, and after prolonged stimulation, and without any of these stimulations (Rudd, et al 2008). Since 2004 when paper III was published, there have been further advances in the understanding of the perforin-dependent cytotoxicity of NK cells and CTLs. It is now well established that perforin is required for the delivery of cytolytic granules, although the precise mechanisms by which this delivery occurs have been debated (Bollito, et al 2007). The fusion of cytolytic granules with the plasma membrane is then mediated through interaction of numerous proteins, among them proteins encoded by UNC 13D and STX11 (Bryceson, et al 2007, Feldmann, et al 2003). Recent studies have shown that patients with mutations in either of these two genes have a defective degranulation, and that the use of flow cytometric assay can demonstrate this defect (Bryceson, et al 2007). In normal NK cells, a transmembrane protein associated with cytolytic granules, CD107a, can be detected on the surface of the cells as they undergo degranulation. In patients with mutations in UNC 13D (FHL type 3) or STX11 (FHL type 4) this expres-
sion of CD107a does not occur, indicating a defective granule exocytosis. FHL type 2 is caused by mutations in PRF1 and therefore characterized by impaired perforin expression whereas cytolytic degranulation is not affected. However a normal expression of perforin does not always exclude mutations in the PRF1 gene (Feldmann, et al 2005). As of today, the diagnostic CD107a assay for degranulation, combined with evaluation of cytotoxicity, offers a rapid method of distinguishing FHL type 2 from types 3 and 4, thereby providing guidance for genetic analysis and classification.

**CNS disease**

CNS involvement is an important contributor of mortality in patients with HLH. An awareness of the occurrence of CNS-HLH and early recognition of its manifestation is therefore of the greatest importance. CNS involvement can dominate the initial course of the disease and precede the systemic symptoms by months. A HLH diagnosis therefore needs to be considered in children with persistent or progressive encephalopathy of “unknown origin”. In general, CNS involvement has been described in the literature as occurring in between 20–73% of the children with HLH (Haddad, et al 1997, Henter and Nennesmo 1997, Janka 1983). This wide range can be attributable to the fact that different reports apply different modalities in describing CNS affection, coupled with a lack of clear definition of CNS disease.

The CNS manifestations of HLH are believed to result from brain infiltration by activated lymphocytes and macrophages. CNS involvement affects both the white and grey matter. Neuropathological studies have demonstrated infiltrations by monocytes and activated lymphocytes in leptomeninges, and brain parenchyma along penetrating vessels (Akima and Sumi 1984, Henter and Nennesmo 1997). A neuropathological grading system has been devised (Henter and Nennesmo 1997). Stage 1 involves the meninges with leptomeningeal infiltrates of lymphocytes and histiocytes only; stage 2 has additional adjacent parenchymal involvement with perivascular infiltration; and stage 3 adds diffuse intraparenchymal infiltrates with multifocal necrosis and reactive astrogliosis, where leukomalacia can be followed by mineralisation. The severity of brain abnormalities is most likely related to the duration of the HLH disease activity.

Data on the frequencies and characteristics of CNS symptoms in large cohorts of patients with HLH have been limited, as has information regarding to what extent CNS involvement at diagnosis can predict long-term outcome. An aim of the HLH-94 study was to better characterize initial and long-term CNS involvement in children with HLH, as well as to analyse the association between
CNS involvement, therapy and prognosis. In paper IV we therefore examined 193 children specifically regarding the relationship between neurological symptoms and/or pathological CSF at start of therapy in relation to long-term outcome.

Initial CNS findings

In paper I, 35/109 of the children (32%) were reported to have neurological symptoms at onset of disease. The frequency of pathological CSF was not investigated. In paper IV neurological symptoms were reported at diagnosis in 72/193 patients (37%, 95% CI 31–44%). The three most common reported symptoms were seizures (33%), irritability (34%) and meningismus (24%). Other symptoms reported were decreased level of consciousness, cranial nerve palsy and ataxia. When interpreting these findings it is important to mention that in HLH-94 there was no standardized neurological evaluation and that neurological examinations in small children can be very challenging. The neurological evaluation of the included patients is probably therefore heterogeneous and the incidence of neurological symptoms at onset underestimated.

Evaluation of CSF is a robust measure. In paper IV the frequency of CSF abnormalities at diagnosis was 101/193 patients (52%, 95% CI 45–59%). This result is in line with a large single-centre study which reported pathological CSF at diagnosis in 22/38 of HLH patients (58%, 95% CI 42–72%) (Mahlaoui, et al 2007) and earlier smaller studies (Haddad, et al 1997, Henter, et al 1991d, Janka 1983).

In relation to the presence of neurological symptoms and/or pathological CSF, our study reflected in paper IV showed a very high overall proportion: 122/193 children (63%). A previous study had found the presence of CNS involvement (using the same definition) in 25/34 (73%) children at time of diagnosis. In addition, that study reported that four of nine patients without CNS involvement at diagnosis later developed a CNS relapse, while the other five either died soon after start of therapy or received an HSCT (Haddad, et al 1997). The authors therefore stated that 100% of their evaluable patients developed CNS lesions in the absence of transplantation. It is possible that most, if not all, patients with FHL will develop some form of CNS involvement if left untreated.

The neuroradiological findings seen in patients with HLH are not specific enough to be diagnostic. However, a spectrum of neuroradiological findings should alert the radiologist to the differential diagnosis HLH. Abnormal neuroradiological findings in children with HLH correlate well with the clinical course of the disease (Goo and Weon 2007). Magnetic Resonance Imaging (MRI) is
considered to be the modality of choice for the diagnosis of CNS complications in HLH patients. The development of new MRI techniques and contrast media offers new possibilities for assessment of perfusion, vascularity, permeability and microcirculation of brain lesions. Diffusion Weighted Imaging can be used to better delineate and even quantify neurodegenerative damage in the brain. Magnetic Resonance Spectroscopic Imaging allows for metabolic mapping for both within and around the lesions, and helps to differentiate on-going active neurodegeneration/inflammation from older lesions and therapy related abnormalities. The combined use of these techniques yields information about lesions’ pathophysiology in vivo, which can improve diagnostic and prognostic abilities and aid in the planning of therapy. However during the study period of paper IV, 1994 to 2003, such advanced neuroimaging techniques as described above were not widely established. Of the 193 patients in paper IV a neuroradiological study was performed in the early phase of the treatment in 115 patients (60%). MRI studies were performed in 75 patients of whom 25 (33%) were found to be abnormal. Computer Tomography investigations were performed in 70 patients of whom 17 (24%) were abnormal. These neuroradiological examinations were not included in the evaluation of CNS findings at onset of disease in relation to outcome for a number of reasons: the examinations were not evaluated in a standardized matter, they were performed in less than half of the study population, and they were performed more often in relation to children displaying clinical CNS symptoms (selection bias). However it is worthy of note that of the pathological neuroradiological findings reported, five were found in patients without any neurological symptoms or pathological CSF at start of therapy. It is therefore possible to conclude that neuroradiological findings may precede apparent clinical findings.

CNS involvement at start of therapy and outcome

Patients with abnormal CSF at diagnosis had a significantly increased risk of mortality if compared to those without (adjusted HR 1.78, 1.08–2.92).

To evaluate if neurological symptoms and/or abnormal CSF had any association with long-term outcome, we divided the patients in paper III into four CNS disease groups: normal CSF and no neurological symptoms (group 1); normal CSF but abnormal CSF (group 2); abnormal CSF but no neurological symptoms (group 3); and abnormal CSF with neurological symptoms (group 4).

None of the patients in CNS group 4 achieved “off-therapy” status. Univariate HR of mortality indicated a significantly reduced survival for both groups 3 and 4 when comparing with group 1. However after adjusting for potential
confounding factors, the adjusted HR only remained statistically significant for group 4 as compared with group 1 (HR 2.05, 1.13–3.72). This finding indicates that there is an increased risk of mortality for patients with both neurological symptoms and abnormal CSF findings when compared with patients with no neurological symptoms and normal CSF.

Late effects
As survival after diagnosis of HLH has improved markedly, it has become increasingly important to thoroughly evaluate long-term involvement. Importantly, neurological dysfunction following HLH may be severe with markedly reduced neurological performance (Haddad, et al 1997, paper I). Despite remarkable improvements in survival rates, few data are available on the long-term clinical status and quality of life of transplanted HLH patients. Since HLH-94 was not designed to specifically evaluate neurological sequelae, the result of neurological late effects should therefore be viewed as more descriptive than comparable.

In paper IV, a substantial proportion of long-term survivors 16/107 (15%), suffered neurological late effects. 102/193 had undergone an HSCT, of whom 67 (66%) were alive at time of analysis. Of these 67, 14 (21%) were reported to have neurological sequelae at their last follow-up. The most common sequelae reported were neurodevelopment retardation (n=7) and epilepsy (n=4). These estimates are higher than what could be expected to be found in the general child population. In addition, attention-deficit/hyperactivity disorder (n=2), hearing loss (n=2), minimal cerebral palsy (n=1) and hemiplegia (n=1) were reported. A higher proportion of neurological late effects in HLH patients surviving HCST has recently been reported (14/39, 36%) (Cesaro, et al 2008). In paper IV neurological sequelae were more frequently reported in CNS group 4 (7/21, 33%) compared with groups 1 to 3 (9/86, 10%) (p= 0.015). Prompt treatment of active CNS-HLH at onset of disease or relapse may reduce these complications as there is evidence that HSCT stabilizes or even improves the late effects (Durken, et al 1999, Shuper, et al 1998). In the study by Cesaro et al none of the 40 patients who were free from CNS manifestations of HLH developed neurological sequelae after HSCT (Cesaro, et al 2008).

Treatment response
Progression of CNS disease constitutes one of the major causes of poor outcome prior to HSCT (paper IV). An important rationale for the intensive initial systemic dexamethasone and etoposide therapy in HLH-94 was an aim to reduce
CNS activity (Henter MPO 1997). In the HLH-94 protocol, the aim was to control CNS disease with systemic therapy before the decision was taken whether intrathecal (IT) MTX therapy should be started or not. If after two weeks of therapy there was clinical evidence of progressive neurological symptoms, or if an abnormal CSF has not improved, additional CNS-therapy is initiated with four weekly IT injections of MTX as well as two additional weeks of systemic high-dose dexamethasone. A similar treatment was also recommended at any time during the course of disease in the event of CNS reactivation.

In paper I neurological alterations were reported in 35 of 109 of the patients (32%) at registration. In these 35 affected individuals, the symptoms had normalized in 21 of 31 survivors (68%) after two months of therapy. The rate of normalization at this time was similar whether IT therapy with MTX was used or not. As IT MTX was not randomized and potentially administered to a group of patients, potentially with selected clinical characteristics the value of additional IT MTX therapy could not be evaluated in this paper. In the study by Haddad et al, similar neurological symptoms developed within months of start of therapy in all evaluable patients treated only with chemotherapy/immune therapy, regardless of the number of IT MTX injections given (Haddad, et al 1997). Another study indicated that the response to this ATG based regimen in patients with neurological disease was worse than for those without neurological disease: The probability of achieving complete remission for patients with neurological disease was 58% (11/19 as compared with those without overt neurological disease (17/19, 89%, p=0.05) (Mahlaoui, et al 2007). As ATG does not cross the blood brain barrier this treatment is probably not very efficient in the treatment of HLH-CNS disease.

Sex

In earlier studies, sex distribution at onset of HLH is known to be more or less equal (Janka 1983, Ishii, et al 1998). Likewise in paper I, there were not significant differences in sex ratio at start of therapy, regardless of whether all patients or familial cases are considered. In paper IV the sex ratio was also equal: 1.1:1.0 (male: female), and no association could be observed between sex and CNS involvement at onset of disease. Turning to survival, in both papers I and IV there was no difference in estimated 3-year probability of overall survival with regards to gender.

In paper I, 23/113 children (20%) were alive without an HSCT, with a median follow-up time ranging from 1.1 to 4.2 years. Within this cohort there were significantly more females (15 F vs. 8 M). Twenty of the 23 children were totally
off therapy and without evidence of disease for more than one year, and were thus considered to be less severe secondary cases of HLH. Of these 20 presumably secondary cases, 13 (65%) were girls.

The sex ratio in children who underwent HSCT was not equal. In paper I, 65 patients were transplanted, with a higher proportion being male (ratio 1.7:1.0, male:female). The proportion of males included in the HSCT study (paper II) was also higher (ratio 1.9:1.0, male:female). Other studies of children transplanted for HLH also displayed a higher proportion of males: in a small study of 14 patients (Jabado, et al 1997) the sex ratio was 1.8:1.0 (male:female); in a study of 20 patients (Baker, et al 1997) the sex ratio was 1.9:1.0 (male:female); and in a study of 48 patients (Ouachee-Chardin, et al 2006) the sex ratio was 1.7:1.0 (male:female). This gender disproportion of more males being allowed for HSCT was not confirmed in a recent study of 91 patients with unrelated donors (Baker, et al 2008). Neither in our HSCT study (paper II) nor in the studies referred above could any association between gender and survival after HSCT be observed.

Age at registration
Earlier studies have shown that 70–80% of children with FHL are below one year of age at onset of the disease with a high proportion of children below six months of age (Arico, et al 1996, Janka 1983). However, since genetic analysis has become available it has been discovered that age at onset of disease may vary, and there are even some cases of FHL that have been diagnosed in adolescence (Allen, et al 2001, Clementi, et al 2002). It is also known that most affected individuals within the same family will have a similar onset (Arico, et al 1996).

In paper I the median age at onset of disease for all 113 patients evaluated was 9 months (range 11 days-12 years). The median age at onset of disease was three months in children with a familial history of FHL and 6 months in patients who received transplants, whereas the corresponding age was higher (29 months) in patients who were alive and off therapy without HSCT for at least one year after start of therapy. In paper III the median age at diagnosis was significantly lower in type 3 patients than in non-type 3 patients (4.8 months vs. 15.3 months, p<0.05 using Mann-Whitney U-test). In addition, when analysing the distribution of cytotoxic activity deficiency sub-types in patients according to age at time of diagnosis, the frequency of patients who were six months old or younger at diagnosis was significantly increased in the type 3 sub-group (21/36, 58%) compared with the non-type 3 group (7/29, 24%) (p= 0.006).

In paper I the overall estimated 3-year probability of survival was signifi-
cantly better in children at least one year old at onset (72% ± 13%) compared to children younger than one year (42% ±12%) (p<0.005 ). This difference did not remain if those patients with presumable secondary HLH (off therapy and alive more than one year after start of therapy) were excluded. In the 25 familial cases, 17 (68%) were younger than one year at start of therapy, and of those eight (47%) were alive at last follow-up. Of the eight familial cases who were older than one year at start of therapy, five (62%) were alive.

In paper II, where 86 children were evaluated after HSCT, the majority (69%) were less than one year old at onset of the disease. In our material, age at onset of disease did not affect survival after an HSCT had been performed. The association of actual age at the time of the HSCT was not analysed. In another study, any association between age at transplantation and survival could not be confirmed (Baker, et al 2008).

PAPER V

FHL is an autosomal-recessive disorder with genetical heterogeneity. The phenotype of FHL can be due to “loss-of-function mutations” in different genes, whereby the gene is disrupted such that no functional protein or a less functional protein is produced. There are currently three genes described in which loss-of function mutations will be causative of FHL: PRF1, UNC13D and STX1 (FHL 2, FHL3 and FHL 4, respectively).

There are various reports on the prevalence of the different mutations, which seems to vary widely in different ethnic groups. As in the case of most other autosomal recessive disorders, FHL appears to be more frequent in countries with traditions of consanguineous marriages. This is due to the so called “founder effect”, meaning that autosomal recessive disorders are more common in inbred populations. In such populations there is a higher than expected chance that two persons carry the same mutated allele.

In paper V we investigated if genotype-phenotype associations could be detected in patients with FHL. Our study cohort included 76 patients, from 65 unrelated families, originating from the Nordic countries, Turkey and the Middle East. Patients were included if their treating physician had evaluated and treated them as having FHL, and if they had been sequenced for the disease causing genes known. A family history of FHL was present in 29/76 children (38%), and 44/75 children (58%) were born to consanguineous parents. The diagnostic criteria of FHL were completely fulfilled in 55/68 patients (81%, no data in eight cases) (Henter, et al 1991b). Due to the high proportion of children who
fulfilled the diagnostic criteria, it could not be expected to find any genotype-phenotype associations regarding these criteria. We could however identify genotype-phenotype associations with regard to ethnic origin, age at onset and pathological CSF at diagnosis.

In all, loss-of-function mutations were diagnosed in 33 of the 76 children with a history of HLH. The majority of the identified mutations had already been described in earlier publications (paper V, Table II). Two bi-allelic mutations and one compound heterozygous mutation were novel observations at the time of the study.

Loss-of-function mutations in PRF1 were found in 13/74 patients (18%), in UNC13D in 6/61 patients (10%) and in STX11 in 14/70 patients (20%). In 27/60 patients (45%) analyzed for all three genes, no molecular diagnosis was established. These patients could carry mutations in genes of FHL that are yet to be discovered, their symptoms could be a result of other forms of primary HLH, or perhaps they could have severe secondary HLH. In 16 patients there was not sufficient DNA to sequence all these three genes, and these patients were therefore not included in the genotype-phenotype association study. To provide a more valid estimate of the prevalence of the different genotypes, the association with ethnicity to genotype was performed based only on unrelated patients (referred to in paper V as families) (n=65).

**Genotype-phenotype associations**

**PRF1 mutations**

PRF1 mutations are reported to have been found all over the world. In our cohort, involvement of PRF1 was demonstrated in 12/63 unrelated families (19%, 95% CI 11–30%). We observed a statistically significantly higher prevalence of PRF1 mutations in families originating from the Middle East compared to families originating from the Nordic countries (6/13 vs. 1/18, p=0.034). This result could also be due to the fact that consanguinity was found to have a strong association with ethnicity, being rare in the Nordic patients. The ethnicity of the patients appear to be associated with specific mutations of PRF1 (zur Stadt et al 2003, Molleran Lee, et al 2004). Trizzano et al could in a study of 63 different mutations of PRF1 show that specific mutations are strongly associated with Turkish, African American and Japanese ethnic groups (Trizzino, et al 2008).

In paper V patients carrying PRF1 mutations were also shown to have a significantly increased risk of an early onset of the disease (less than six months of age) compared with patients carrying mutations in STX11. The association remained after adjusting for ethnicity as a potential confounding factor (OR
Age at onset, may be associated with disease severity (paper I), and has also been shown to correlate with type of PRF1 mutation. In a Japanese study of 40 unrelated patients, 11 showed PRF1 mutations (28%, 95% CI 16–43%). The onset of three FHL 2 patients with missense mutations was late (7, 11 and 12 years) (Ueda, et al 2006). Similarly, a genotype-phenotype study of 14 unrelated children with PRF1 deficiency concluded that the only manifestation that differed between missense and nonsense mutations was age at diagnosis, which showed a higher variability in the missense group (Feldmann, et al 2002).

In our study, three patients carrying missense mutations also displayed a wide range in age at onset (range 0.9–61.5 months). In a comprehensive genotype-phenotype study of specific mutations in PRF1 these findings were confirmed: patients with two disruptive mutations were statistically significantly younger at onset of the disease compared with patients with missense mutations only (p<0.001) (Trizzino, et al 2008).

When we compared the four genotype groups, we were not able to identify any associations with PRF1 mutations and disease status at two months after start of therapy. The clinical outcome is described as being associated with the presence of residual perforin or absence of perforin in the cytotoxic cells (Molleran Lee, et al 2004). In a study by Trizzano et al, nonsense mutations were significantly associated with absent NK cytotoxicity. Patients with two disruptive mutations had a significantly lower NK cytotoxicity than patients with either a combination of one disruptive and one missense mutation, or patients with two missense mutations (p=0.008) (Trizzino, et al 2008).

**UNC13D mutations**

In our cohort the prevalence of FHL 3 in unrelated families was 4/50 (8%, 95% CI 3–19%). None of these families were of Nordic origin. This prevalence is similar to that reported for German patients (zur Stadt, et al 2006) but is lower than that reported from Italian and Japanese studies (Santoro, et al 2006, Yamamoto, et al 2004).

A high frequency of patients with UNC13D mutations and CNS involvement has been reported from France (9/10, 90%, 95% CI 60–98%) (Feldmann, et al 2003) and Italy (9/15, 60%, 95% CI 36–80%) (Santoro, et al 2006). In paper V, pathological CSF was chosen as an indicator of CNS involvement of the disease. In our cohort, 2/5 patients with UNC13D mutations presented with neurological symptoms and 3/5 had pathological CSF at start of therapy. No firm conclusions can be drawn on associations with phenotype due to the small number of patients with UNC13D mutations in our study.
STX11 mutations

STX11 mutations were initially only reported from patients originating from Turkey (Rudd, et al 2006, zur Stadt, et al 2006). Later on, patients from the Middle East have also been reported (Bryceson, et al 2007). In paper V bi-allelic STX11 mutations were present in 8/69 unrelated families (12%, 95% CI 6–21%), all of whom were of either of Turkish or Middle Eastern origin, and in no case of Nordic origin. A statistically significant difference in the frequency of STX11 mutations was noted among Turkish families compared to Nordic families (7/28 vs. 0/17, p=0.034). zur Stadt et al also showed that STX11 mutations were found in significantly more patients in patients with Turkish origin than those with German origin (zur Stadt, et al 2006). In a Japanese study, no STX11 mutations were found (Yamamoto, et al 2005).

A significant difference in age at onset was identified in patients carrying STX11 mutations compared to PRF1 mutations, with the FHL 4 patients being older (more than six months) at diagnosis. The statistical calculation is described above. Of the 14 patients with STX11 mutations, 7 (50%) were older than one year at diagnosis. It has been shown by in vitro studies that patients with FHL4 (and FHL3) can restore their cytotoxic deficiency. When stimulated with IL-2, the cells from patients carrying STX11 mutations showed a normal degranulation pattern as compared with healthy, age-matched controls (Bryceson, et al 2007). If the same would be true in vivo, that IL-2 acts as an innate rescue mechanism in the early phase of the disease, this would then explain the delayed onset in some patients with STX11 mutations.

Early onset of disease is associated with an increased mortality (paper I), as is pathological CSF (paper III). In paper V there was a significantly decreased risk of pathological CSF in patients carrying STX11 mutations compared to the group where none of the genes had been identified (see below). None of the patients with STX11 mutations were less than six months and had pathological CSF at diagnosis. The corresponding results for patients with PRF1 mutations and no identified mutation was 5/11 patients (45%, 95% CI X) and 6/20 (30%, 95% CI x), respectively. Our findings in paper V thus suggest that patients with STX11 mutations might have a low frequency of early onset and pathological CSF, which could indicate a less severe disease.

No mutations identified

We were not able to establish any molecular diagnosis in 27 of 60 patients (45%), corresponding to 25/49 unrelated families (51%, 95% CI 37–64%). These patients may carry mutations in one of the known genes, although we failed to de-
tect them. It could also be these patients harbour yet unknown genetic defects. Of the patients with no mutation identified 7/27 (26%) had a family history of FHL and 8/27 (30%) were born to consanguineous parents.

For the majority of patients originating from the Nordic countries no mutation was identified in any of the known disease-causing genes. In the families of Nordic origin the prevalence of “no identified mutation” was significantly higher compared both with families from Turkey (13/14 vs. 10/24, p<0.002), and with families from the Middle East (13/14 vs. 2/11, p<0.001). This is consistent with a study including patients of German origin where no mutation could be found in 16/23 patients (70%, 95% CI 49–84%).

Patients without identified mutations had increased risk of pathological CSF at diagnosis compared with patients with *STX11* mutations (adjusted OR 26.37, 1.90–366.82). This finding may indicate that patients without identified mutations carry mutations in yet unknown genes that encode proteins important for the development of CNS-HLH. Further search for additional causative genes is warranted.
In order to perform a meaningful clinical study on a rare disease, a collaborative international effort is required. The multi-centre study HLH-94 provides a successful example of such an effort. This study made it possible to create a database where all patients, treatments and clinical outcomes were registered. The database is the largest international database on children treated for HLH, currently including approximately 500 patients. This registry is based at the Childhood Cancer Research Unit at Karolinska Institutet in Stockholm, Sweden. Treatment according to the HLH-94 protocol has led to a dramatic increase in survival, and the work presented in this thesis has had a great impact on the treatment of children with HLH worldwide. Papers I and IV represent the two largest prospective studies of children treated for HLH so far.

In 2004 a new treatment protocol was launched by the Histiocyte Society. This new protocol, HLH-2004 (Henter, et al. 2007), was based on the achievements and results made in HLH-94 (paper I) with minor revisions. It was thought that treatment intensity should be increased during the first two months of therapy with a drug that does not induce myelotoxicity. Therefore in HLH-2004 treatment with CsA commences at the start of therapy instead of after eight weeks as in the HLH-94 protocol. For treatment of CNS disease, IT steroids were added to IT MTX. Currently HLH-2004 is the most widely used treatment protocol for patients with HLH worldwide. When these treatment changes were introduced it would have been preferable to randomize between the old and new protocol. This was not possible however due to the limited number of patients with HLH. One concern is that there still are patients who do not respond to the HLH-2004 therapy and for these non-responders efficient treatment regimens are currently missing. Further improvements in the treatment of this disease are therefore needed.

It is of great importance to better define and predict CNS disease in HLH. This would help improve the treatment leading to greater survival and less late effects. Our findings in paper IV stress the importance of carrying out full CNS work-up by the use of clinical neurological examination, CSF investigation and neuroradiology. Of these three, only CSF investigation was sufficiently standardized in HLH-94, whilst neurological examination could be more subjective. The new treatment protocol, HLH-2004, addresses this by requiring specific information about neurological symptoms. However, even greater standardization of these procedures would be beneficial.

The outcome post HSCT may be influenced by patient characteristics, do-
nor characteristics and transplant related factors. In a multicentre study with wide heterogeneity the outcome may be influenced by a transplant centre effect and local population characteristics, thus potentially limiting the precision of some of our findings.

In paper III we showed an association between NK cytotoxicity subtype and the possibility of coming “off” therapy. We are aware that there might be competing risks for patients coming off therapy and those receiving transplantation. If the HCST is performed very quickly after start of therapy then the child will not have the possibility to come “off” therapy. However, only two of the 34 transplanted children had HSCT less than four months from start of therapy.

Phenotypes are complex and often variable and studies of genotype-phenotype associations can be difficult to conduct. However findings of genotype-phenotype associations that are reproducible in populations from different backgrounds can be valuable and our findings in paper V have been replicated. A clear link between mutation and prognosis can provide better genetic advice to affected families. It is also expected that in the future other disease causing genes will be identified. New techniques are being developed for the identification of disease-causing mutations. An objective standardised description of the phenotype, despite potential instability over time, can make optimal use of these advanced methods by determining the clinical impact of mutations in potential candidate genes.

In paper I, II and IV strict inclusion criteria for HLH were applied to avoid misclassification of patients who were reported as treated on the protocol but perhaps did not have FHL. A limitation of the studies is that the assessment of neurological symptoms and disease activity may be somewhat subjective. Despite its lack of specificity, disease activity at two months after start of therapy is a useful measure since it was conducted at the same time point in all patients, providing the basis for a meaningful comparison between patients.

One of the limitations of our study population (paper I-IV) is that it includes children from many different centres. There may be differences in the associations studied because of variation between centres and populations. Further confounding may arise through differences in rapidity of diagnosis and treatment. Unfortunately there was insufficient statistical power to adjust for each treating centre. It is also difficult to estimate the possible effect of selection-bias in this heterogeneous multicentre study.

The number of patients with missing data in paper I-III is minimal. In paper IV there were 44 patients with missing criteria. However, there were no
differences in outcome or pattern at presentation detected when these 44 were compared with the 193 studied. **Paper V** included 76 children but only 59 were studied with regard to the association with early age, and only 46 with regard to the association with pathological CSF. If those children with missing CSF data comprised a selected group, with either more or less severe disease, their exclusion may have biased the results.

Despite the heterogeneity of the studies and the limitations described, clear conclusions can be drawn from the research covered by this thesis.
CONCLUSIONS

The work in this thesis has been performed with the aim of contributing to an improvement in the survival of children with HLH. In conclusion, the first four papers relating to patients treated according to the HLH-94 protocol, including HSCT, revealed that:

- HLH-94 has contributed to a greatly improved survival of children with HLH, and around half of the patients with familial HLH are long term survivors.
- HLH-94 has been used effectively in a wide range of institutions internationally.
- Two thirds of the transplanted patients are long-term survivors.
- Survival for MUD transplant recipients is equal to survival for MRD transplant recipients.
- Transplants using Haploidentical donors and MMUD can result in an acceptable outcome if no other donor is available.
- Patients with non-active disease after induction therapy fared best, and active disease at transplantation should not automatically preclude transplantation, although this needs to be investigated in future studies.
- Classifying NK cell cytotoxicity deficiency subtypes may be a valuable tool for determining if an HSCT should be performed.
- 63% of patients with HLH had neurological symptoms and/or abnormal CSF at disease onset.
- 15% of long-term survivors had neurological sequelae.
- Children with both neurological symptoms and abnormal CSF had an increased risk of mortality compared with patients with no neurological symptoms and normal CSF at diagnosis.

Furthermore, our studies of genotype-phenotype associations revealed that:

- The frequency of gene mutations varies with ethnicity.
- The disease-causing mutations in FHL display different phenotypes with regard to age at onset and pathological CSF at diagnosis.
SPECULATION AND FUTURE PERSPECTIVES

Appropriate diagnosis and treatment is necessary for survival in patients with FHL or with severe secondary HLH. At present, the unfortunate situation is that these diagnoses may be missed or delayed in many patients worldwide. This can be due to ignorance as to the existence of the syndrome, or inappropriate diagnosis. It is important that information about this disease and its treatment is widely distributed, particularly since treatment is life-saving in a high proportion of children.

As there are still children who fail to be cured of the disease, further studies are needed to improve the existing treatment protocols. It would be beneficial to find early prognostic markers in order to identify those children who do not respond to treatment. To achieve this, more extensive assessments of initial status and associated outcomes are recommended. More use of internationally accepted specific disease criteria as well as relapse criteria would allow for future meta-analyses.

To evaluate treatments, it would be of benefit to conduct prospective randomized trials between the two treatment protocols which are currently used (HLH-2004 and ATG based therapy). However, the rate at which new patients are currently registered is not sufficiently high to conduct such a trial within a reasonable period of time. Another element which would improve treatment is salvage therapy which needs to be identified for this disease.

Further studies of the biology underlying the disease in the patients who fail to respond may be valuable. It could be that these patients have a more severe form of the disease, in which case a more intensive treatment might be indicated. An alternative speculation is that these patients may have developed resistance to chemotherapy, although this has not been shown to date.

With regard to CNS disease, a future study of CNS disease in patients with HLH is being planned. This HLH-CNS study will be carried out as a prospective study with the aim of creating a clear and internationally applicable definition of CNS disease in HLH, as well as to identify the relevant associated outcomes. This would allow a standardised diagnostic protocol to be created and implemented. Ultimately, the aim is to improve treatment for affected children.

It has been noted that a high proportion of children with HLH die post-HSCT. Further studies are therefore required to investigate why this is the case. To what extent does disease activity at the time of transplantation, in terms of identifiable individual disease markers, affect outcome? What constitutes optimal pre-transplant conditioning? New reduced intensity conditioning regimens
are increasingly used in non malignant diseases and will have to be evaluated, as well as the role of unrelated cord blood transplants in HLH. Furthermore, in a monogenic disease such as FHL, studies could be conducted into the use of gene-therapy as an alternative therapy.

The development of a biological understanding of HLH, as well as the vastly improved treatment-outcome, are significant success stories. Although HLH is a very rare disease, studies of this disorder has also contributed to greater understanding of disturbed immune homeostasis in a wider context. I hope that this progress will continue, and that I can be an active part of the journey.
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