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**GENETIC, CELLULAR AND CLINICAL STUDIES OF
HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS**

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Front cover – Natural Killer cell (left) killing a target cell (right). Courtesy of Stephanie Wood.

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Bona diagnosis, bona curratio

ABSTRACT

Hemophagocytic lymphohistocytosis (HLH) is a life-threatening hyperinflammatory condition characterized by fever, cytopenia, hepatosplenomegaly, and sometimes hemophagocytosis. HLH is typically divided into two distinct groups, primary HLH and secondary HLH. Familial HLH (FHL), of autosomal recessive inheritance, is divided into type 2, 3, 4, and 5, caused by aberrations in *PRF1*, *UNC13D*, *STX11*, and *STXBP2*, respectively; all encoding proteins involved in the perforin-mediated cytotoxic pathway. Consequently, patients with FHL display a defective NK cell cytotoxicity, one of the diagnostic criteria for HLH. The clinical presentation of the different forms of HLH can vary markedly, and the distinction between primary and secondary HLH is not always clear.

In 1991, Henter *et al.* reported the first estimate of the annual incidence of FHL. Based on an increased awareness of HLH, with available diagnostic guidelines and increased clinical and biological understanding, we, in **Paper I**, hypothesized that the true incidence could be higher than previously estimated. However, somewhat surprisingly, the estimated annual incidence of primary HLH in Sweden was unchanged, 1.2 per million children less than 15 years of age, corresponding to 1.8 per 100 000 live born children. The annual incidence in patients aged less than 1 year was 11 per million children. The second part of **Paper I** aimed to provide a minimal incidence of primary HLH based on genetic findings and NK cell function consistent with primary HLH. Using these methods, twelve such patients referred to us were identified 2007-2011, giving a minimal incidence of 1.5 per million children aged less than 15 years in Sweden, corresponding to 2.2 patients per 100 000 live born children. These laboratory diagnostic tools may facilitate the diagnosis of primary HLH.

In many patients with primary HLH, especially in patients of Scandinavian origin, the underlying molecular defect has not been identified. Thus, one of the major aims with this thesis was to provide a genetic diagnosis for these patients. Patients with Griscelli syndrome type 2, another autosomal recessive immunodeficiency associated with development of HLH, display a partial albinism in addition to the immunological defect. In **Paper II**, one out of 21 families diagnosed as having FHL, was identified with bi-allelic mutations in *RAB27A*, and thus instead affected by GS2. Three additional GS2 patients were also first diagnosed as having FHL and first later diagnosed with GS2, further stressing the importance of remembering GS2 among patients with HLH. The partial albinism in GS2 patients may easily be overlooked. In **Paper III**, Rab27a was shown to be required for NK cell cytotoxicity and degranulation induced by receptors both for natural cytotoxicity and antibody dependent cellular cytotoxicity, in contrast to what previously has been described. Furthermore, recruitment of Rab27a and Munc13-4 to perforin-containing granules was shown regulated by different receptor signals, with an inverse relationship between Rab27a and Munc13-4.

In **Paper IV**, we describe the clinical presentation, the mutation spectrum, and NK cell function in patients with FHL type 5. Interestingly, a highly variable disease severity was observed among these patients, with an age at onset ranging from 2 months to 17 years. Furthermore, gastrointestinal symptoms, bleeding disorders, and hypogammaglobulinemia were present in about one third of the patients. Thus, we conclude that the clinical presentation of FHL type 5 can vary markedly, and that FHL5 should be considered also in patients with manifestations not typically associated with FHL. In **Paper V**, two non-coding aberrations in *UNC13D* were described to be causative of FHL type 3 in many patients of European origin, highlighting that aberrations outside the coding regions also can be a cause of disease. The first is a point mutation in intron 1 that selectively impairs *UNC13D* transcription in lymphocytes and the second is a 253-kb inversion straddling the *UNC13D* locus that affects the 3' end of the transcript. Both aberrations abolish the Munc13-4 expression.

Taken together, this thesis provides genetic, cellular, and clinical findings of importance for the understanding of HLH. A genetic diagnosis, together with assessment of cytotoxic lymphocyte function, facilitates the diagnosis of patients with primary HLH and enables presymptomatic identification of affected individuals. Furthermore, a genetic diagnosis enables carrier testing and prenatal diagnosis in the affected families. Studies of cytotoxic lymphocyte function in these patients also provide fundamental insights in lymphocyte cytotoxic function and human immunology. A genetic diagnosis, together with an increased knowledge about the diverse clinical presentation, is highly valuable in the clinical management and for prompt initiation of adequate treatment in these life-threatening immunodeficiencies.

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- I. **Meeths M**, Horne AC, Sabel M, Bryceson YT, Henter JI. Incidence of primary hemophagocytic lymphohistiocytosis in Sweden.
Manuscript.
- II. **Meeths M**, Bryceson YT, Rudd E, Zheng C, Wood SM, Ramme K, Beutel K, Hasle H, Heilmann C, Hultenby K, Ljunggren HG, Fadeel B, Nordenskjöld M, Henter JI.
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- III. Wood SM, **Meeths M**, Chiang SC, Bechensteen AG, Boelens JJ, Heilmann C, Horiuchi H, Rosthøj S, Rutynowska O, Winiarski J, Stow JL, Nordenskjöld M, Henter JI, Ljunggren HG, Bryceson YT.
Different NK cell-activating receptors preferentially recruit Rab27a or Munc13-4 to perforin-containing granules for cytotoxicity.
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- IV. **Meeths M**, Entesarian M, Al-Herz W, Chiang SC, Wood SM, Al-Ateeqi W, Almazan F, Boelens JJ, Hasle H, Ifversen M, Lund B, van den Berg JM, Gustafsson B, Hjelmqvist H, Nordenskjöld M, Bryceson YT, Henter JI.
Spectrum of clinical presentations in familial hemophagocytic lymphohistiocytosis (FHL) type 5 patients with mutations in *STXBP2*.
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- V. **Meeths M**, Chiang SC, Wood SM, Entesarian M, Schlums H, Bang B, Nordenskjöld E, Björklund C, Jakovljevic G, Jazbec J, Hasle H, Holmqvist BM, Rajic L, Pfeifer S, Rosthøj S, Sabel M, Salmi TT, Stokland T, Winiarski J, Ljunggren HG, Fadeel B, Nordenskjöld M, Henter JI, Bryceson YT.
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LIST OF ABBREVIATIONS

ADCC	antibody dependent cell-mediated cytotoxicity
APC	antigen presenting cells
ATG	antithymocyte globulins
C3	complement component 3
CHS	Chediak-Higashi syndrome
CRAC	calcium release-activated calcium
CSF	cerebrospinal fluid
cSMAC	supramolecular activation complex
CSR	class switching recombination
CTLs	cytotoxic T cells
DAG	diacylglycerol
DCs	dendritic cells
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic
FADD	Fas-associated death domain
FBS	fetal bovine serum
FHL	familial hemophagocytic lymphohistiocytosis
GS2	Griscelli syndrome type 2
HLH	hemophagocytic lymphohistiocytosis
HPS2	Hermansky-Pudlak syndrome type 2
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
ICAM	intercellular adhesion molecule
ICD	International Classification of Diseases
IFN	interferon
IHGSC	International Human Genome Sequencing Consortium
IL	interleukin
IRF3	interferon response factor 3
ITK	IL-2-inducible T cell kinase
KIR	the killer cell Ig-like receptors

KLR	killer cell lectin-like receptors
LCMV	lymphocyte choriomeningitic virus
LPS	lipopolysaccharide
LU	lytic units
LYST	lysosomal trafficking regulator
MAC	membrane-attack complex
MACPF	membrane-attack-complex/PRF
MAS	macrophage activation syndrome
MHC	major histocompatibility complex
MRI	magnetic resonance imaging
MTOC	microtubule organizing center
NADPH	nicotinamide adenine dinucleotide phosphate
NFκB	nuclear factor κB
NGS	next-generation sequencing
NK cells	Natural Killer cells
OMIM	Online Mendelian Inheritance in Man
PAMPs	pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PCR	polymerase chain reactions
PGD	preimplantation genetic diagnosis
PID	Primary immunodeficiency
PMA	phorbol 12-myristate 13-acetate
pSMAC	peripheral supramolecular activation complex
RAG	recombination activating gene
RIC	reduced-intensity care conditioning
RNA	ribonucleic acid
SAP	SLAM associated protein
SCID	severe combined immunodeficiency
SCN	severe congenital neutropenia
SLAM	signalling lymphocytic activating molecule
Slp3	synaptotagmin-like protein 3
SNARE	soluble N-ethylmaleimide-sensitive factor activating protein receptor
SNP	single nucleotide polymorphism
TCRs	T cell receptors

TLRs	toll-like receptors
TNF	tumor necrosis factor
TRIF	TIR-domain-containing adapter-inducing interferon- β
VAMP	vesicle-associated membrane protein
WBCs	white blood cells
XIAP	X-linked inhibitor of apoptosis
XLP	X-linked lymphoproliferative syndrome

FOREWORD

This thesis comprises studies of a group of inherited immunodeficiencies termed primary hemophagocytic lymphohistiocytosis (HLH). HLH is characterized by a disproportionate immune response and a severe systemic hyperinflammation. Without adequate treatment of HLH the prognosis is poor. My aim and hope when I started working with this thesis was to be able to improve the diagnostic work-up of severely sick children affected by HLH. It is rewarding that this aim now has been accomplished. An early and correct diagnosis is important for prompt initiation of adequate treatment and thus also for an improved outcome.

The first part of the introduction aims to provide a general background about human immunology and human genetics for readers outside the field. This part is followed by a more specific introductory section about HLH and mechanisms of lymphocyte cytotoxicity, as well as a discussion about the findings and concluding remarks and future perspectives. The last section includes the five papers comprising the thesis.

Stockholm, September 9, 2012

Marie Meeths

1 INTRODUCTION

1.1 HUMAN IMMUNOLOGY

1.1.1 The immune system

We are living in a hostile world with a continuous need for protection, and for survival our immune system is absolutely crucial. The different components of the immune system are defending us from invading pathogens and malignantly transformed or damaged cells. The immune system is complex and strictly regulated, in order to exert its function and still avoid self-damage. However, in spite of a functional immune system, we are still vulnerable to different infections. Infection-related mortality has over the years been high, and the increase in life expectancy, due to improved hygienic procedures, development of antimicrobial drugs and vaccination, is relatively recent.

Primary immunodeficiencies (PIDs) have previously been considered as rare Mendelian disorders resulting in fatal infections during infancy. However, the last decade the knowledge of PIDs has increased considerably, and the underlying genetic defects have now been identified in many previously disparate syndromes associated with increased susceptibility to infection. The identification of the molecular defects has also resulted in identification of novel PIDs. There is currently an on-going debate about the definition of PIDs and today PIDs are also recognized as a cause of more subtle disease presentation [1, 2]. JL Casanova has introduced the concept of monogenic “holes” in immunity that cause susceptibility to specific infectious disease in otherwise healthy individuals [3], and the question is if “inborn errors of immunity to infection” can be considered “the rule rather than the exception” [4].

1.1.2 Hematopoietic stem cells

The white blood cells are generated from a common progenitor, the pluripotent hematopoietic stem cell (HSC). This process, called hematopoiesis, is restricted to different parts of the body during different ages. In the embryo, the hematopoiesis first occurs in the yolk sac, later in the liver, and thereafter in the spleen. In the fourth or fifth fetal month, the hematopoiesis starts to shift to the bone marrow, and at birth the bone marrow is the main location for the hematopoiesis [5].

The HSCs are by definition self-renewing, undifferentiated cells that can give rise to all mature blood cells, of both lymphoid lineage (T cells, B cells, and Natural Killer cells (NK cells)) and myeloid lineage (erythrocytes, megakaryocytes/platelets, monocytes, macrophages, dendritic cells (DCs), granulocytes). HSCs are rare, counting for only one in thousand of the cells in the bone marrow [6]. Even though the HSCs are the most studied stem cell in humans, the complexity of these cells is not yet fully understood. It has been shown that HSCs constitute different subsets with fixed epigenetically determined differentiation and self-renewal programs; lymphoid biased HSCs, myeloid biased HSCs, and balanced HSCs. All types possess self-renewal

capacity and an ability to differentiate into all different types of mature cells, thus fulfilling the criteria for a pluripotent stem cell [7, 8].

1.1.3 Basic overview of the innate and adaptive immunity

The immune system is traditionally divided into two separate entities; the innate immunity and the adaptive immunity. The innate immune system conducts the initial immune response, with germ-line encoded receptors for recognition of targets. These receptors, evolved by natural selection, are limited in their diversity and recognize pathogen-associated molecular patterns (PAMPs), instead of specific disease-causing agents, pathogens, such as a virus, bacterium, or fungus. In contrast, T and B cells, mediators of the adaptive immunity, have an extreme diversity of receptors generated by somatic recombination. The term ‘antigen’ originally came from ‘antibody generator’ and means a substance that can induce an immune response by binding to the B cell receptor, an antibody, or to the T cell receptor. The cells with a receptor that specifically binds to an antigen can undergo clonal expansion, inducing a specific and efficient immune response. In contrast to the innate immune response, the adaptive response takes several days to develop on the first encounter of a pathogen. An important feature of the adaptive immune system is the immunological memory, favorable upon a second encounter of the specific pathogen.

1.1.3.1 *Innate immunity*

The innate immunity is characterized by fixed germ-line encoded receptors and effector molecules [9]. The physical barriers, including the skin and the mucosa, are the first line of defense against invading pathogens. In addition to the anatomical barrier, a diversity of antimicrobial agents, including peptides called defensins, are secreted from all epitheliums [10]. Damage to the barriers, enable the entry of pathogens into the body’s soft tissue.

The complement system consists of serum proteins, produced in the liver [11]. They initiate a cascade of enzymatic reactions resulting in the cleavage of complement component 3 (C3) to C3a and C3b. C3b becomes covalently bound to the pathogen, and functions as a recognition-tag for phagocytic cells, a mechanism called opsonization. C3b is also involved in the formation of the membrane-attack complex (MAC), which forms a pore in the plasma membrane and induces cell lysis in the pathogen. C3a is implicated in recruitment of phagocytes [11]. The complement system is divided into three different pathways; the alternative pathway, the lectin pathway, and the classical pathway. The classical pathway is implicated in both the innate and the adaptive immune response, which includes binding of antibodies to the pathogens’ surface. All three pathways converge at C3, finally ending up with the MAC complex, C5b-9 [11]. Of note, the structure of C9 in the MAC complex shows similarities to perforin, a pore-forming protein involved in lymphocyte cytotoxicity [12]. Defects in the complement system can lead to autoimmune manifestations or infections, such as the susceptibility to neisserial infections seen in patients with a deficiency in the MAC complex [13].

Monocytes are the circulating precursors of macrophages and dendritic cells (DCs). While migrating from the circulation into the tissues, they are differentiated into DCs and macrophages, professional phagocytic cells [14]. Macrophages have a variety of receptors recognizing signals not normally found in healthy tissues. Engagement of the receptors induces phagocytosis and destruction of the engulfed pathogen in a compartment called the phagolysosome [14]. The phagocytic capacity is enhanced by opsonization, coating of the pathogens by complement or antibodies. Toll-like receptors (TLRs) are a family of receptors that recognize different types of PAMPs [9, 15]. Ten different TLR genes are so far identified in humans [9, 15]. Different family members of the TLRs respond to different microbial products, e.g., lipopolysaccharide (LPS), double stranded ribonucleic acid (RNA) (present in many viral infections), unmethylated CpG nucleotide motifs (abundant in bacterial and viral but not in human genomes), zymosan (derived from yeast cell walls) [5]. Together the TLRs recognize a large variety of microorganisms. TLR4 expressed by macrophages recognizes LPS, and are thus important in the defense against Gram-negative bacteria [9, 15]. Signaling through the TLR4 leads to recruitment of the adaptor proteins MyD88 or TIR-domain-containing adapter-inducing interferon- β (TRIF) [9, 15]. Recruitment of MyD88 results in binding to IRAK-4 and activation of the transcription factor nuclear factor- κ B (NF- κ B), important for both the innate and adaptive immune responses by upregulation of genes encoding pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, CXCL8 (also known as IL-8), IL-12 and tumor necrosis factor- α (TNF- α) [16]. Signaling through the TRIF pathway results in activation of the transcription factor interferon response factor 3 (IRF3) and production of antiviral cytokines called type 1 interferons [16]. The TRIF pathway is also activated by the binding of microbial products, like double-stranded RNA components of some viral genomes, to TLR3 [5]. Defects in protein involved in TLR-mediated signaling result in a susceptibility to different specific pathogens [17]. Patients with defects in *MyD88* and *IRAK-4* suffer from severe and invasive bacterial infections [18, 19], whereas patients with herpes simplex encephalitis have been reported with inherited defects in the TLR3-immunity [20].

Neutrophils are the most abundant white blood cells, circulating in the blood awaiting signals to be recruited to the site of infection. They are phagocytic cells that, in contrast to the macrophages, have a short lifetime of about two days. They are stored in the bone marrow for up to five days before being released into the peripheral blood. Neutrophils have receptors recognizing microbial products and opsonized pathogens. After engulfment and fusion of the phagosome with preformed neutrophil granules, different toxic substances and enzymes eliminate the pathogen. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is essential in the elimination of the pathogen [5]. Individuals without functioning neutrophils are susceptible to fungal and bacterial infections [17]. Patients with chronic granulomatous disease have reduced neutrophil function due to defects in the NADPH-complex whereas severe congenital neutropenia (SCN) includes a heterogeneous group of disorders characterized by low neutrophil counts [17, 21, 22]. In addition to the susceptibility to bacterial and fungal infections, patients with SCN have an increased risk of leukemia-development. The cumulative incidence of myelodysplastic syndrome/leukemia in SCN patients has been reported to be 31 % [21].

NK cells were first described 1975 as large granular lymphocytes, encompassing cytotoxic capacity [23, 24]. NK cells comprise 15 % of the circulating lymphocytes [25]. They are defined as cells of the innate immunity, with a key role in the defense against intracellular viral and bacterial pathogens, as well as neoplastic cells. In addition to their cytotoxic capability of killing their target cells, they are important cytokine secreting cells [26]. They are in the early stage of an infection the main producers of IFN- γ , a type II interferon [26]. IFN- γ activates the macrophages to secrete cytokines, initiating the adaptive immune response. Their ability to kill their targets is enhanced by activation by interferons, such as IFN- α and IFN- β induced by viral infections [26]. Type I interferons also induce proliferation of the NK cells. IL-12 is a potent activator of IFN- γ production in NK cells [26]. NK cells also play an important role in the control of expansion of autologous immune cells [27]. Of interest, several reports of NK cell-mediated adaptive immunity have been published the last decade, providing evidence for NK cell memory [28]. Patients with NK cell-deficiency are susceptible to viral infections, especially to the herpesvirus and papillomavirus families [29].

1.1.3.2 Adaptive immunity

The adaptive immunity is characterized by a highly specific, adaptable, and efficient immune response, which, in contrast to the innate immune system, takes several days to be fully functioning upon the first encounter of a specific pathogen. The adaptive immunity can be divided into the cell-mediated and the humoral immunity, conducted by T cells and B cells, respectively. The receptors for antigen recognition are all of the same molecular type and are highly specific in contrast to the receptors in the innate immunity. The receptors are encoded by genes allowing rearrangement leading to production of a huge variety of receptors [30-32].

Immature B cells are produced in the bone marrow. B cells are characterized by expression of the B cell receptor, an immunoglobulin, specifically recognizing the antigens. The immunoglobulin consists of two heavy chains and two light chains. They have a variable region binding the antigen, and a constant region that is very similar between the different immunoglobulins. For the soluble immunoglobulins the constant region binds to Fc receptors on phagocytes and cytotoxic cells, inducing antibody-mediated phagocytosis and antibody-dependent cellular cytotoxicity (ADCC), respectively. The T cell receptors (TCRs) are always membrane-bound, and consist of an α -chain (TCR α) and a β -chain (TCR β), or alternatively, in a minority of cases, a γ -chain and a δ -chain. Similarly to the B cell receptor, the TCRs have a variable region for specific binding of the antigen, and a constant region.

The variable regions of immunoglobulins and TCRs are encoded by gene segments called V, D, and J. The heavy chain gene in the immunoglobulin and the β -chain gene in the TCR have arrays of V, D and J segments, whereas the light chain and the α -chain only include V and J segments. An enzyme-catalyzed process, where different combinations of the segments are brought together, called rearrangement, is required to acquire a functional gene. This process takes place in the somatic cells and is called somatic recombination [5]. Two of the enzymes involved in the rearrangement are

recombination activating gene (RAG)-1 and RAG-2, expressed only in T cells and B cells [33]. These enzymes are essential for the adaptive immunity, and the RAG genes and the adaptive immunity are thought to have arisen in ancestors of jawed vertebrates approximately 500 million years ago [34].

The somatic recombination results in a diversity of specific receptors and production of millions of different variants of circulating lymphocytes. Upon infection by a pathogen, only a small subset of the lymphocytes will have receptors recognizing the specific antigen. The adaptive immune response is initiated in specialized lymphoid tissues such as lymph nodes, spleen, and Peyer's patches in the intestines. This promotes the meeting of the small numbers of lymphocyte with the specific antigens, but requires presentation of the pathogen by different antigen presentation cells (APCs) such as the DCs. Upon binding to a naïve T-cell with a receptor specific for the antigen (clonal selection), the T cell starts to proliferate to produce a clone with the specific receptor (clonal expansion). Upon proliferation, they differentiate into different effector lymphocytes with specific capacities [5].

The antigen recognized by the TCRs are short peptides presented by the APCs. The peptide is generated by degradation of the pathogen proteins in the APCs and presented bound to a major histocompatibility complex (MHC) molecule. There are two types of MHC molecules, MHC class I and MHC class II. MHC class II molecules present antigens from extracellular pathogens, and is expressed by APCs. MHC class I is expressed by almost all cells and presents antigens from intracellular pathogens.

Cytotoxic T cells (CTLs) are activated by engagement of the TCR. In addition, binding of the co-receptor CD8 to a conserved site on the MHC I molecule is necessary for target cell killing of infected cells [5]. The helper T cells instead express the co-receptor CD4, which binds to a conserved site of the MHC class II molecule. T helper cells enhance the phagocytosis of extracellular pathogens by macrophages and neutrophils either by direct contact with macrophages or by secretion of activating cytokines. Similarly, binding of or cytokine secretion by T helper cells is essential for differentiation and production of antibodies by the B cells.

When the activated B cells proliferate, somatic hypermutation of the immunoglobulin takes place, producing immunoglobulins that bind the pathogen more tightly than the original B cell receptors [35]. B cells with strong binding are chosen for becoming plasma cells, effector cells specialized in antibody production. Class switching recombination (CSR) is another mechanism for a more efficient response that changes the constant region of the immunoglobulin but does not involve the variable antigen-binding site [36]. Five different isotypes of immunoglobulins are produced, IgA, IgD, IgE, IgG, and IgM. IgM and IgD are cell surface antigen receptors on B cells. The main secreted antibodies are IgM, IgA and IgG. CSR enables production of the preferable isotype depending on requirements of the immune response for a specific pathogen [37]. Defects in the production of antibodies are associated with an increased susceptibility to bacterial infections, particularly affecting the upper respiratory tract [17]. X-linked agammaglobulinemia is the most common form of early onset agammaglobulinemia. Common variable immunodeficiency disorders, with reduced

levels of one or more subclasses of antibodies, is the most common clinically significant PID presenting mainly in adult age [17].

Immunological tolerance towards self is needed to avoid self-damage, and includes mechanisms to avoid T cells with receptors recognizing self-antigens. Positive selection takes place in the thymus, and identifies thymocytes (immature T cells) carrying TCRs that efficiently bind to MHC molecules. Those T cells not binding MHC molecules undergo apoptosis. Negative selection is the mechanism where the surviving T cells that bind too strongly to self-MHC undergo apoptosis. A similar mechanism of negative selection of B cells takes place in the bone marrow. However, since the B cells need the T helper cells for the proliferation the necessity of self-tolerance is not as critical. Another mechanism for self-tolerance is regulatory T cells, which suppress responses of self-reactive T cells [38].

The clonal expansion of pathogen-specific B and T cells also lead to production of memory cells allowing for an immunological memory that can last for life. One of the advantages with the adaptive immune system is that a second encounter of the same pathogen results in a much faster and more efficient immune response.

Severe combined immunodeficiency (SCID) comprises disorders with genetically determined defects in T cell differentiation together with variable defects in the differentiation of other lineages [39]. SCID is classified based on the immunological phenotype with absence of T cells but not B cells or absence of both T and B cells. Both groups include forms with or without NK cells [17]. SCID can be of both X-linked and autosomal recessive inheritance. The patients present early in life with life-threatening infections, chronic diarrhea, and failure to thrive [17]. Thymic defects, including DiGeorge syndrome, may also result in T cell deficiency [17]. Defects in the cell-mediated cytotoxicity include both the innate and the adaptive immune system, and will be described in more detail later.

1.2 HUMAN GENETICS

1.2.1 Basic overview

In 1865, Gregor Mendel published the seminal paper ‘Experiments on Plant Hybridization’ upon which the discipline of classical genetics was founded [40]. In this paper he described inherited characteristics in plants, with one trait being dominant to the recessive alternative, today called the Mendelian inheritance. The paper was not highly recognized initially, but was re-discovered in the early 1900s.

In 1882, another German scientist, Walther Flemming, identified thread-like structures in the nucleus of the cell, later named chromosomes [41]. Humans have 23 pairs of chromosomes in the cell nucleus, of which 22 pairs are autosomes and one pair is sex chromosomes. In each pair, one chromosome is inherited from each parent. Each chromosome consists of a single deoxyribonucleic acid (DNA) molecule. In 1944, Oswald Avery, Colin McLeod and Maclyn McCarty reported DNA to constitute the hereditary material in bacteria [42]. The DNA molecule consists of polymers of the

nucleotides adenine, cytosine, guanine, and thymine, forming two strands running in opposite directions. This double-helix structure of DNA was first described by Watson and Crick in 1953 [43]. To fit in the cell nucleus, the DNA double-helix is packed together with histones and other proteins forming a structure called chromatin. The human genome consists of about 20 000 to 25 000 genes [44], which are the functional units of DNA encoding a protein or RNA. The composition of RNA is similar to DNA, except that RNA contains ribose in contrast to DNA that contains deoxyribose. Furthermore, thymine is replaced by uracil in RNA. The DNA is transcribed into RNA, which after post-transcriptional modifications is translated into a chain of amino acids, forming proteins. The post-transcriptional modifications include 5' capping, 3' polyadenylation, and RNA splicing. Splicing is the mechanism in which non-coding sequences, introns, in the gene are removed. The genetic code describes how a set of three nucleotides, a codon, is translated into a specific amino acid. Upon cell division the genome is duplicated by replication of the DNA molecules, providing 46 chromosomes to each daughter cell.

Mutations are variants in the DNA caused by mistakes in the replication or by environmental factors. If a mutation arises during meiosis, it can be inherited by the offspring. This individual will carry the mutation in all cells, called a constitutional mutation. The children of a carrier of a constitutional mutation can, in turn, inherit the mutation. In opposite, a somatic mutation arises only in the somatic cells, and will not be passed on.

A mutation causative of disease is called a pathogenic mutation. A mutation can involve large segments of DNA, e.g., large duplications, deletions, or inversions, or smaller fragments e.g. small deletions or insertions, or single base pair exchanges. The small deletions and insertions can result in loss or gain of amino acids and/or cause shifts in the reading frame during translation. A mutation of a single base pair, a point mutation, introducing a stop codon in the reading frame is called a nonsense mutation. A missense mutation is a point mutation resulting in a change of a codon to one coding for a different amino acid, whereas a silent mutation does not result in a change of the amino acid. Certain genetic variants are more common in the population, called polymorphisms. A single nucleotide polymorphism (SNP) is an exchange of a single nucleotide, existing at different allele frequencies in different populations.

1.2.2 Mendelian diseases and inheritance patterns

A Mendelian trait, or disease, is controlled by a single locus, and follows the rules of Mendelian inheritance in contrast to multifactorial diseases. For each trait, one gene variant is inherited from each parent. The alternative gene variants are called alleles. If the alleles are identical they are called homozygous, and if they are not identical they are called heterozygous.

Mendelian diseases can be autosomal or X-linked, depending on the location of the gene responsible for the disease. A disease is dominant if heterozygous carriers display the phenotype, and recessive if only homozygous or compound heterozygous (different mutation on the two alleles of a gene) carriers are affected. Furthermore, mutations can

have different penetrance (the proportion of mutation carriers of the genotype that develop the phenotype), and expressivity (the degree to which the genotype is phenotypically expressed). The “Online Mendelian Inheritance in Man” (OMIM), is a database of human genes and genetic disorders.

1.2.3 Sequencing of the human genome

The human genome-project was initiated 1984 with the primary role to determine the sequence of the human genome. In 2001 two separate drafts of the human genome were published, by the International Human Genome Sequencing Consortium (IHGSC) and by the privately funded Celera Genomics, respectively [45, 46]. In 2004 the IHGSC reported the results of the finished project [44], and in 2007 the first sequence of an individual human genome was published [47]. The last years there has been a shift from conventional Sanger sequencing to newer methods referred to as next-generation sequencing (NGS) [48]. NGS platforms are now widely available and the cost of DNA sequencing is constantly decreasing, allowing for production of an enormous amount of sequencing data. The 1000 genomes project is the first project to sequence a large number of people and has the goal to identify most of the variants with a frequency over 1 % in the populations studied. The results of the pilot-phase of the study were published in 2010 [49]. Sequencing of only the coding regions of the genome, constituting less than 2 %, is termed whole-exome sequencing. This approach reduces the costs and has been proven a useful tool in discovering Mendelian disease genes [50].

1.3 HEMOPHAGOCYTIC LYMPHOHISTOCYTOSIS

1.3.1 History, classification, and diagnostics

James W. Farquhar and Albert E. Claireaux in 1952 described two children with a rapidly fatal disease, none of them diagnosed before death. Farquhar and Claireux diagnosed these children with what they named familial hemophagocytic reticulosis [51]. The term familial hemophagocytic reticulosis was later changed to familial hemophagocytic lymphohistiocytosis (FHL). One year earlier, in 1951, Reese and Levy had reported two sisters diagnosed with Letter-Siwe disease, which both died in infancy with fever, cytopenia, hepatosplenomegaly, hemophagocytosis. Retrospectively this probably represent the first siblings with FHL reported in the medical literature [52].

The clinical presentation of hemophagocytic lymphohistiocytosis (HLH) is characterized by prolonged fever, hepatosplenomegaly, and cytopenia [53-55]. Other common findings include high fasting triglycerides, low fibrinogen, high ferritin, hepatitis/acute liver failure, elevated sCD25, and hemophagocytosis [53-55]. Furthermore, neurological involvement with neurological symptoms and/or pathological cerebrospinal fluid (CSF) is common, and is, importantly, sometimes the first symptom [56-59]. Of note, the presentation of HLH can vary markedly between different patients.

HLH is typically divided into two different entities, primary HLH and secondary HLH. The primary form is caused by inherited genetic aberrations, whereas there is no clear inheritance pattern of secondary HLH. Secondary HLH can be triggered by an underlying infection, a malignancy, or a rheumatological disorder. These different types are termed infection-associated HLH, malignancy-associated HLH, and rheuma-associated HLH, respectively. The latter is also often called macrophage activation syndrome [60]. The distinction between primary and secondary HLH is not always clear.

As the clinical presentation of HLH is diverse, there is often a delay in making the diagnosis. To guide the treating clinicians, the first diagnostic guidelines for HLH were published in 1991 by Henter *et al.* [61]. These criteria were also used in the first international treatment protocol, HLH-94 (Table 1) [62]. The currently used criteria were published together with the updated treatment protocol, HLH-2004 (Table 1) [63].

Table 1. Diagnostic criteria for HLH according to HLH-94 and HLH-2004 [62, 63].

	HLH-94	HLH-2004
<i>Fever</i>	5 of 5 criteria or a molecular diagnosis.	5 of 8 criteria or a molecular diagnosis
<i>Splenomegaly</i>		
<i>Bicytopenia</i>		
<i>Hemoglobin (Hb) <90 g/L (infants <4 weeks: Hb <100 g/L)</i>		
<i>Platelets <100×10⁹/L</i>		
<i>Neutrophils <1.0 ×10⁹/L</i>		
<i>Elevated triglycerides and/or decreased fibrinogen</i>		
<i>Fasting triglycerides ≥3.0 mmol/L (i.e., <265 mg/dl)</i>		
<i>Fibrinogen ≤1.5 g/L</i>		
<i>Hemophagocytosis in bone marrow, spleen or lymphnodes</i>		
<i>No evidence of malignancy</i>		
<i>Ferritin >500 mg/L</i>		
<i>sCD25 (i.e., α-chain of soluble IL-2 receptor) >2,400 U/ml</i>		
<i>Defective NK cell function (according to local laboratory)</i>		

Criteria for the diagnosis of HLH according to HLH-94 and HLH-2004, respectively. In addition, a positive familial history, and consanguinity are suggestive of HLH according to HLH-94 [62, 63].

1.3.2 Genetics in FHL

In 1999, the first two genetic loci associated with FHL were reported back-to-back [64, 65]. FHL1 (9q21.3-22, Table 2) was identified by homozygosity mapping of four inbred families of Pakistani origin [65]. The disease-causing gene has so far not been identified in this locus, whereas Stepp *et al.* later the same year described *PRF1* as the causative gene of FHL2 (Table 2) [66]. *PRF1* encodes perforin, involved in cytotoxic lymphocyte function. In 2003, the FHL3 locus was mapped to 17q25 in 10 patients from 7 unrelated families and *UNC13D* (Table 2), encoding Munc13-4, was identified as the causative gene by Feldmann *et al.* [67]. Genome-wide homozygosity mapping in a large consanguineous family of Kurdish origin revealed linkage to 6q24, defining the

FHL4 locus, and homozygous mutations were identified in *STX11* (Table 2) in this and other families in 2005 [68]. Lastly, two different research groups in 2009 assigned FHL5 to 19p13, and the fourth gene, *STXBP2* (Table 2), causative of FHL was identified [69, 70]. *STX11* and *STXBP2*, encode syntaxin 11 and Munc18-2, respectively. The function of these four proteins will be described in the section entitled ‘Mechanisms of lymphocyte cytotoxicity’.

1.3.3 Primary hemophagocytic syndromes

In addition to FHL with an autosomal recessive inheritance, primary hemophagocytic syndromes also include other syndromes.

Griscelli syndrome type 2 (GS2) and Chediak-Higashi syndrome (CHS) are both autosomal recessive primary immunodeficiency syndromes associated with development of HLH in addition to a partial albinism. GS2 and CHS are caused by mutations in *RAB27A* and *LYST*, respectively (Table 2) [71, 72]. Patients with CHS have leukocyte cytoplasmatic giant-granules, not present in GS2 patients [71, 73]. In addition, patients with CHS have been described with a bleeding tendency and neurological impairments. The neurological symptoms in CHS may progress also after a hematopoietic stem cell transplantation (HSCT) [74].

HLH has also been described in a patient with Hermansky-Pudlak syndrome type 2 (HPS2), another autosomal recessive primary immunodeficiency with partial albinism, caused by defects in *AP3B1* (Table 2) [75, 76]. Of note, the patient described also carried a heterozygous mutation in *RAB27A*, and was first suspected to have GS2. Other symptoms associated with HPS2 are bleedings, recurrent infections, and neutropenia [77, 78].

X-linked lymphoproliferative syndrome (XLP) is a primary immunodeficiency characterized by vulnerability to Epstein-Barr virus (EBV), frequently complicated by development of HLH [79]. XLP is divided into two different subtypes, XLP1 and XLP2, caused by mutations in *SH2D1A* and *XIAP*, respectively (Table 2) [80, 81]. In both groups of patients, HLH episodes are often triggered by an EBV-infection [82]. Hypogammaglobulinemia has been documented in XLP1 and XLP2 patients, however to a greater extent in the XLP1 patients [82]. About one third of XLP1 patients develop lymphoma in contrast to none of the reported XLP2 patients [82]. In patients with XLP2, splenomegaly without fulminant HLH and colitis have both been reported [82]. SAP, encoded by *SH2D1A*, is a regulator of NKT cell development resulting in a lack of NKT cells in patients with XLP1, which is not observed in XLP2 patients [83, 84]. Furthermore, an impaired development of memory B cells is seen in XLP1 patients and the patients fail to produce an isotype class switched immune response after vaccinations [85].

Mutations were recently identified in IL-2-inducible T cell kinase (*ITK*, Table 2) in two sisters with a clinical picture resembling XLP, including EBV positive B cell proliferation following EBV-infection [86]. In contrast to the genes causative of XLP, *ITK* is an autosomal gene. Thus ITK-deficiency can affect both males and females.

Table 2. Genes associated with primary HLH.

	Causative gene	Locus	Year of identification of the causative gene/locus
FHL			
FHL1	Unknown	9q21.3-22	1999
FHL2	<i>PRF1</i>	10q22.1	1999
FHL3	<i>UNC13D</i>	17q25.1	2003
FHL4	<i>STX11</i>	6q24.2	2005
FHL5	<i>STXBP2</i>	19p13.2	2009
Other immunodeficiencies often associated with HLH-development			
GS2	<i>RAB27A</i>	15q21.3	2000
CHS	<i>LYST</i>	1q42.3	1997
HPS2	<i>AP3B1</i>	5q14.1	1999
XLP1	<i>SH2D1A</i>	Xq25	1998
XLP2	<i>XIAP</i>	Xq25	2006
ITK-deficiency	<i>ITK</i>	5q33.3	2009

1.3.4 Secondary hemophagocytic lymphohistiocytosis

Secondary HLH can complicate an underlying disease in both children and adults. Even though secondary HLH is not a Mendelian disorder, underlying genetic defects predisposing for HLH might be revealed in the future.

Among infection-associated HLH, EBV is the most commonly reported triggering pathogen, especially in Asia [87]. Other viruses identified as triggers of HLH are human herpes virus 6, cytomegalovirus, adenovirus, parvovirus, and varizella-zoster virus [87]. In addition, bacteria, fungi, and protozoae can also trigger HLH [87]. A noteworthy trigger of HLH is Leishmaniasis, reported in many cases [88]. However, a triggering pathogen does not rule out primary HLH [89].

Malignancy-associated HLH has most commonly been reported in patients with lymphomas and leukemias of T-cell or NK-cell lineages, but can also be associated with anaplastic large cell lymphomas, B lineage lymphoblastic leukemias, myeloid leukemias, mediastinal germ cell tumors, and other solid tumors [90]. Of importance, some patients develop HLH before the underlying malignancy is detected [91].

Macrophage activation syndrome (MAS) is a severe, sometimes life-threatening complication to rheumatological diseases, especially systemic-onset juvenile idiopathic arthritis, adult-onset Still's disease, and systemic lupus erythematosus [55]. MAS patients may present with all characteristics of HLH, and MAS has been suggested to instead be termed secondary HLH [60].

1.3.5 Pathogenesis and pathophysiology

HLH is a hyperinflammatory condition characterized by a disproportionate immune response. Identification of the underlying genetic defects in patients with HLH and the increased knowledge about the function of the encoded proteins, have led to a better understanding of the pathogenesis and pathophysiology of HLH. All the known genes causative of FHL encode proteins involved in lymphocyte cytotoxicity. Of note, defective lymphocyte cytotoxicity was described in FHL patients already in 1978 [92], and was suggested a diagnostic criterion for HLH in 1984 [93]. It was included in the diagnostic criteria in the HLH-2004 protocol [21].

FHL is characterized by an excessive proliferation and activation of CTLs upon triggering of the immune system. The CTLs secrete pro-inflammatory cytokines, which activates macrophages. Tissues are infiltrated by these polyclonal CTLs and macrophages, and the activated macrophages phagocytose blood cells, called hemophagocytosis. In FHL patients, the feedback mechanism with down regulation of the immune response via perforin-dependent cytotoxicity is defective, allowing the hyper-inflammatory state to continue. Patients with secondary HLH do not have permanently defective lymphocyte cytotoxicity. However, they can have a transient defect, such as due to low numbers of cytotoxic cells.

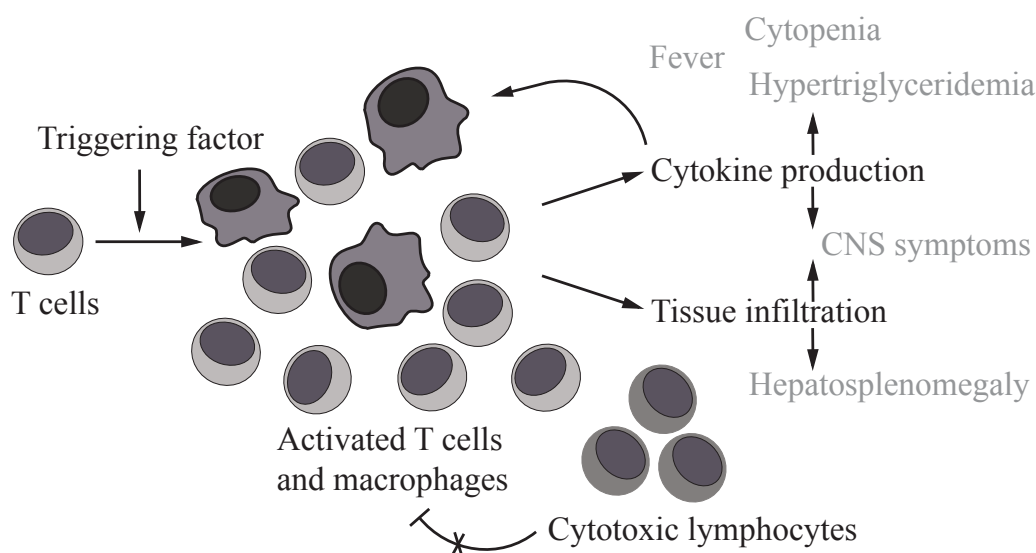
The pronounced hypercytokinemia in patients with HLH was first described in 1991 [94]. The elevated levels of IFN- γ , TNF- α , and IL-6 suggested an important role of pro-inflammatory cytokines in the pathophysiology of HLH. Other cytokines e.g., IL-10, and macrophage colony-stimulating factor, have later also been described elevated in HLH patients [95, 96]. In perforin-deficient mice, an animal model of HLH, IFN- γ was shown to be essential for development of the HLH features upon infection with lymphocyte choriomeningitic virus (LCMV) [97]. The IFN- γ production appeared to be driven by increased antigen presentation to the CD8⁺ T cells [97]. It was later revealed that blocking of IFN- γ had a therapeutic effect of HLH in LCMV infected perforin-deficient mice, as well as in Rab27a-deficient mice, the animal model of GS2 [98].

1.3.6 Clinical and laboratory features

The tissue infiltration of lymphocytes and macrophages together with the hypercytokinemia explain many of the clinical and laboratory features of HLH (Figure 1). Fever can be induced by the increased levels of the pyrogenic cytokines IL-1, IL-6, and TNF- α [29]. The cytopenia is secondary to inhibitory effects of cytokines on the hematopoiesis, in addition to the hemophagocytosis of activated macrophages [97, 99, 100]. The activated macrophages secrete ferritin and plasminogen activator, resulting in the elevated levels of ferritin and decreased levels of fibrinogen seen in patients with HLH [55]. The hypertriglyceridemia observed in many patients can be attributable the suppression of lipoprotein lipase activity by inflammatory cytokines such as TNF- α [101, 102]. Soluble CD25 (sCD25, the α -chain of the interleukin 2-receptor), typically elevated in HLH patients, is upregulated on activated T cells [103]. The hepatosplenomegaly is a result of tissue infiltration of activated T cells and macrophages. Neuropathological studies of patients with CNS involvement have

revealed lymphocyte and monocyte infiltration, which may secrete damaging cytokines and neurotoxic factors [56, 59].

Figure 1. Schematic view of the pathophysiology of primary HLH.



1.3.7 Therapy and outcome

1.3.7.1 Chemo-immunotherapy

HLH can be rapidly fatal, and without treatment the median survival in FHL is between one and two months after diagnosis [104, 105]. The estimated 5-year survival was 17 % in a report of 122 patients published in 1996 [106]. These patients were not treated according to any common consensus protocol for HLH. Successful treatment including podophyllotoxin derivatives (teniposide and etoposide) and corticosteroids was first reported in the 1980s [107-109]. Later on, cyclosporine A was reported to be useful in the continuation treatment of primary HLH [110].

The first prospective international treatment protocol, HLH-94, was developed by the Histiocyte Society and opened in 1994. The inclusion criteria in the study are described on page 9 (Table 1) [62]. The induction therapy in HLH-94 was based on etoposide, dexamethasone, and, in selected patients, intrathecal methotrexate [62]. The continuation therapy included etoposide, dexamethasone pulses, and cyclosporine A, and started after the 8-week induction [62]. A HSCT was recommended for patients with a familial disease, preferable with an HLA-identical donor if available [62]. HLH-94 was closed in the end of 2003, and the long-term results of the study were published last year [111]. In total 249 patients were eligible for inclusion. After the initial treatment of 2 months, 214 patients (86 %) were alive, and 122 of 207 (59 %) of these patients were reported with non-active disease [111]. The 5-year probability-of-survival

was reported to be 54 ± 6 % [111]. Overall, 124 (50%) patient underwent HSCT after a median time of 6.1 months [111].

The HLH-2004 protocol was based on the experience from the HLH-94 protocol, aiming to further improve the outcome. In addition to etoposide and dexamethasone, cyclosporine A was included in the induction therapy [63]. Patients with a familial history, known genetic diagnosis, or with severe, persistent or reactivated HLH were recommended continuation therapy, and for these patients HSCT was recommended as early as possible [63]. Patients without a known familial history that responded well to treatment with complete resolution were recommended to be put off treatment and closely followed in regards to HLH parameters [63]. The HLH-2004 study was closed end of 2011. The HLH-2004 study was closed end of 2011 and the results of the study are yet not published.

The relationship between clinical findings and early pre-transplant deaths were studied in 232 patients treated with either HLH-94 or HLH-2004 [112]. Hyperbilirubinemia, hyperferritinemia, and cerebrospinal fluid pleocytosis at diagnosis were identified as risk factors for an adverse outcome, and trombocytopenia and hyperferritiemia were risk factors 2 weeks into therapy [112]. Moreover, the treatment of GS2, CHS, and XLP with HLH-94 and HLH-2004 has been evaluated in a small cohort [113]. The results suggest that HLH-directed therapy is effective in inducing remission of HLH in these syndromes [113].

Results of an alternative treatment regimen of FHL, including antithymocyte globulins (ATG), used in a single-center study of 38 patients were reported 2007 [114]. A combination of ATG, corticosteroids, cyclosporine A, and intrathecal methotrexate were used. The therapy led to rapid and complete response in 73 % of the cases, and partial response in 29 % [114]. One patient did not respond to treatment at all and ten patients relapsed, whereof nine subsequently died [114]. In total, 79 % patients underwent HSCT and the overall survival was 55 % [114]. The efficacy of the ATG-based regimen and HLH-94 is difficult to compare since no randomized has been performed. However, the overall survival is essentially similar, 55 % and 54 %, respectively. Notably, while the ATG study was performed in one single experienced center, HLH-94 was a multinational study [62, 114]. A trial using a “hybrid” immunotherapy combining ATG, corticosteroids, and etoposide is currently open.

In addition to the above mentioned treatment regimens, alemtuzumab has been suggested a therapeutic role in the treatment of refractory HLH and rituximab has been used in EBV-associated HLH and XLP [115-117]. Furthermore, IFN- γ blocking antibodies have been suggested a putative therapeutic role in HLH, based on the function of IFN- γ in the pathophysiology described above [98, 118].

For reliable comparison between the different treatment regimens, randomized studies are needed. Since the number of HLH patients is limited, international collaboration is vital for recruitment of a sufficient number of patients.

1.3.7.2 Hematopoietic stem cell transplantation

The first allogeneic HSCT for a HLH patient was reported with a favorable outcome in 1986 [119]. Further strengthening the role of HSCT in the treatment of FHL, an increased estimated 5-year survival for patients that had undergone a HSCT compared to those without (66 % compared to 10 %) was seen in 122 patients [106].

In the HLH-94 protocol the conditioning regimen and graft-vs-host disease prophylaxis were determined by the different transplantation units. The outcome of HSCT in patients treated with the HLH-94 protocol has been evaluated by Horne *et al.* [120]. Altogether 86 patients were included in that study, with a median age of 13 months at HSCT. The 3-year over-all survival post-HSCT was 64 % \pm 10 % [120]. Sub-divided into different donor groups the 3-year survival was 71% \pm 18 % for matched related donors, 70 % \pm 16 % for matched unrelated donors, 50 % \pm 24 % for familial haplo-identical donors, and 54 % \pm 27% for mismatched related donors [120]. Based on this, the use of alternative donors at experienced centers was suggested when matched donors are unavailable [120]. Thirty-one patients were deceased at the point of analyses, whereof 26 were reported with transplant-related mortality, and two with relapse of HLH. Of note, patients with a better response to the pre-transplant induction therapy had a better outcome.

In the evaluation of the ATG-based regimen the post-HSCT survival was 74 % [114]. Among patients treated with the ATG-based regimen as the first-line therapy the outcome was better, 84 % (n = 19) compared to 50 % (n = 8) of patients treated with the ATG-therapy as second-line treatment [114]. Of note, all patients in this study were treated at the same, highly experienced center, as compared to those in the multi-center HLH-94 study. The median time between onset of therapy and HSCT was 6 weeks (range 4-32 weeks) [114].

Two different studies, with twelve and 26 patients respectively, indicate a favorable outcome with reduced-intensity care conditioning (RIC) regimen compared to myeloablative conditioning [121, 122]. In one study, the estimated 3-year survival was 92 % \pm 11 % for RIC patients and 43 % \pm 26 % for myeloablative conditioning patients [121, 122]. The other study reported 84 % of the RIC patients to be alive and well after HSCT with a median follow up of 36 months [121, 122]. Mixed donor-recipient chimerism was more frequent in RIC patients than myeloablative conditioning patients, 65 % compared to 18 %. The only patient relapsing in that report did so after the whole blood donor chimerism fell to <10 % [122]. Another study of HSCT in HLH, describe sustained remission for up to 20 years in all patients with a donor chimerism > 20 % [123].

With regard to disease activity at HSCT, Horne *et al.* reported that many patients survived HSCT despite active HLH at the time of transplant, and that HSCT therefore not automatically should be precluded in HLH patients with active disease [120]. These findings were later supported by another study, which reported acceptable survival in all patients groups except those with both active disease and haploidentical donors [123]. Nevertheless, it is still strongly suggested, if possible, to perform HSCT in a state of remission.

1.4 MECHANISMS OF LYMPHOCYTE CYTOTOXICITY

CTLs and NK cells are cytotoxic lymphocytes important in the immune response to intracellular pathogens, and malignantly transformed cells, and for the immune homeostasis. Cytotoxic lymphocytes kill their target cells via the perforin-mediated pathway, involving directed secretion of secretory lysosomes, as well as, to some extent, death receptor signaling. The perforin-mediated pathway for lymphocyte cytotoxicity is schematically illustrated on page 21 (Figure 2).

1.4.1 Secretory lysosomes

A defining factor for mature cytotoxic lymphocytes are the secretory lysosomes. These are specialized lysosomal organelles, containing the secretory proteins perforin and pro-apoptotic granule serine proteases (granzymes) in addition to the standard lysosomal proteins [124]. NK cells have pre-formed secretory lysosomes, whereas T cells need stimulation by the TCR to induce expression of the cytotoxic proteins [124, 125]. Of importance to avoid self-damage, the low pH in the secretory lysosomes prevents the cytotoxic function of perforin [126]. Furthermore, perforin has a calcium-dependent C2 domain at the C-terminus, mediating plasma membrane binding and cytotoxic activity. The higher calcium-level in the extracellular space is needed for the cytotoxic function [126]. The storage of secretory proteins in dense cores of the secretory lysosomes can protect them from degradation by other lysosomal proteins [124]. The secretory lysosomes also contain membrane-bound FasL, the ligand for the death receptor Fas. Polarized degranulation of the secretory lysosomes upon recognition of the target cells controls the delivery of the soluble cytotoxic proteins to the extracellular space between the effector cell and the target cell, as well as the delivery of FasL and TRAIL to the cell surface [124, 127].

The protein lysosomal trafficking regulator (LYST) contains a BEACH (Beige and Chediak-Higashi)-domain, and is implicated in the function of secretory lysosomes. Different studies have indicated a role for LYST in the sorting and trafficking of proteins (Figure 2) [128]. Patients with bi-allelic mutations in *LYST* (CHS patients) have typical giant intracytoplasmic lysosomal structures in different cell types [74]. Clones of CTLs from CHS patients express normal levels of the soluble lytic proteins perforin and granzyme A and B, but show a defective capacity in the exocytosis of secretory lysosomes [129].

The protein AP3 has a suggested role in the transport of proteins from the trans-Golgi network, to endosome- or lysosome-related organisms (Figure 2) [130]. CTLs from a patient with a homozygous mutation in the gene *AP3B1*, encoding one of the sub-units of AP3, have been described with a higher base-line level of LAMP1 (CD107a) [75, 131], enlarged secretory lysosomes, and defective polarized secretion of the secretory lysosomes [131].

1.4.2 Target cell recognition

Upon specific antigen binding of the TCR, naïve T cells requires several days for proliferation and acquisition of effector function, whereas NK cells are ready to kill with their pre-formed, perforin-containing secretory lysosomes. The antigen-specific TCRs is generated by somatic recombination during T cells development as described above.

In contrast to CTLs, NK cells express a set of germ-line encoded receptors, which control activation, proliferation, and effector function [132]. As a consequence of the pre-formed cytotoxic granules, the cytotoxic function needs to be highly regulated in NK cells, with a fine-tuned balance between activating and inhibitory receptors [132, 133]. Activation via receptors for natural cytotoxicity, by binding to ligands on the target cell, can directly initiate target cell killing. NKp46, NKp30, NKp44, NKG2D, 2B4, and NKp80, are all activation receptors for natural cytotoxicity [133, 134]. NKp46, NKp30, NKp44, and 2B4 belong to the immunoglobulin superfamily whereas NKG2D and NKp80 belong to the C-type lectin receptor family [94]. In addition, NK cells express the low-affinity Fc-receptor IIIA (CD16) for recognition and killing of IgG coated target cells by ADCC [135]. The inhibitory receptors consists of two families; the killer cell Ig-like receptors (KIR) and the killer cell lectin-like receptors (KLR), which recognize MHC class I expressed by almost all cells and prevent killing of the normal cells [134]. An important mechanism is that lack of MHC I molecules on the target cells signals for killing, called the “missing self hypothesis” [136].

Target cell killing is controlled by engagement of different receptor signals for polarization and degranulation [137]. Binding of LFA-1 to its ligand intercellular adhesion molecule (ICAM)-1 induce polarization, but not degranulation, whereas engagement of CD16 by IgG induce degranulation but not polarization of the secretory granules in NK cells [137]. For target cell killing, engagement of both LFA1 and CD16 is required [137]. Binding of 2B4 by its ligand CD48 result in weak polarization but no degranulation, whereas concurrent activation of 2B4 and CD16 result in polarization and killing, even without engagement of LFA1 [137]. 2B4 is a member of the signaling lymphocytic activating molecule (SLAM)-family and can either stimulate or inhibit NK cell activation via cytoplasmatic immunoreceptor tyrosin-based phosphorylated switch motifs. These motifs can recruit phosphatases for negative regulation or SLAM-associated protein (SAP) for positive regulation. Binding of SAP leads to association with the Src kinase FynT, and protein tyrosine phosphorylation signals [138]. In SAP deficient cells, CD2 or CD16 induced NK cell cytotoxicity has been shown to be normal, whereas stimulation of 2B4 failed to induce cytotoxicity (Figure 2) [139].

1.4.3 Immunological synapse

Target cell recognitions activate down-stream signaling and trigger a number of events resulting in exocytosis of the secretory lysosomes. First, an immunological synapse is formed between the effector and the target cell through which the secretory granules can be exocytosed and delivered to the target cell (Figure 2).

In T cells, the TCRs are involved in forming the central supramolecular activation complex (cSMAC), which is surrounded by integrins (e.g., LFA-1) forming the peripheral SMAC (pSMAC) involved in the cell-cell adhesion [140-142]. However, the necessity of the formation of a stable synapse for cytotoxicity in CTLs has been questioned [143]. After activation, coordinated with the movement of the centrosome towards the synapse, actin accumulates at the synapse, and is reorganized to form a ring around the pSMAC, called the distal SMAC [141, 144]. Docking of the centrosome, microtubule organizing center, occurs next to the cSMAC, and directs the movement of the secretory granules towards the synapse [141]. The same mechanism has been observed in NK cells, resulting in delivery of the secretory lysosomes to a single focus spot at the immunological synapse [142].

A recent study of the NK cell cytotoxic synapse has shown that the integrin LFA-1 directs the organization and the transport at the immunological synapse. During degranulation, exocytosed LAMP-1 (CD107a) was accumulated in a central region of the synapse, where bi-directional vesicle traffic takes place [145].

1.4.4 Exocytosis

Exocytosis of the secretory lysosomes involves calcium-dependent signaling. Target cell recognition produces a signaling cascade which activates PLC γ 1, mediating generation of IP $_3$ and diacylglycerol (DAG) from PIP $_2$ [146]. The IP $_3$ stimulates Ca $^{2+}$ release into the cytoplasm from the endoplasmic reticulum, triggering extracellular Ca $^{2+}$ influx through calcium release-activated calcium (CRAC) channels [146]. Activation of CRAC channels are dependent on STIM1 [146]. The store-operated Ca $^{2+}$ entry of extracellular Ca $^{2+}$ is crucial for exocytosis of the secretory granules and lymphocyte cytotoxicity [147]. DAG is accumulated at the immunological synapse [16], and drives the MTOC polarization towards the synapse in T cells [148, 149]. Engagement of LFA-1 in NK cells leads to TCR ζ -chain phosphorylation, SYK, and PLC- γ activation [150]. Other proteins involved in the LFA-1 induced polarization of secretory lysosomes are talin and the Wiscott-Aldrich protein [151].

The secretory lysosomes move along the microtubules towards the microtubule organizing center, in a minus-end directed transport mediated by the protein dynein. Myosin IIa has been demonstrated a function in the vesicle movement close to the synapse [152]. Kinesin-1, a mediator of plus-end-directed microtubule dependent transport, was recently described necessary for the terminal transport of the secretory lysosomes, in a complex with synaptotagmin-like protein 3 (Slp3) and Rab27a, suggesting a switch to ante-grade polarization before the final myosin IIa-dependent transport on actin and fusion with the plasma membrane [153]. Granules in Rab27a-deficient cells have been shown to fail to dock at the synapse (Figure 2) [154, 155].

Before the fusion with the plasma membrane occurs, the secretory lysosomes have to be primed. Munc13 proteins are involved in the priming step at synapses [156, 157]. Munc13-4 contains two C2 Ca $^{2+}$ -binding domains suggesting a Ca $^{2+}$ -dependent function. Exocytosis of the docked granules at the synapse is defective in Munc13-4

deficient cells, indicating a role of Munc13-4 in the priming of the vesicles before the fusion event in the immunological synapse (Figure 2) [67].

Soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins are implicated in the fusion event. A functional SNARE complex requires formation of a four-helix bundle between the fusing membranes. In the four-helix bundle, four distinct motifs must be represented; R-, Qa-, Qb-, and Qc-SNARE motifs [158]. The R-SNARE is usually vesicle associated, whereas Q-SNAREs are target-SNAREs. Most of the SNARE proteins have one SNARE motif each, whereas SNAP-proteins have two SNARE-motifs. The composition of the SNARE complex required for the fusion event in the immunological synapse is not fully elucidated. Vesicle-associated membrane protein (VAMP)-7 and VAMP-8 have both been suggested as candidates for the R-SNARE motif, but knockdown experiments did not completely reduce the target cell killing [159, 160]. Patients with absent or defective syntaxin 11 display defective exocytosis and target cell killing, that is partially restored by IL-2 stimulation [161]. Munc18-2 in complex with syntaxin 11 is suggested to have a role in the fusion event by regulating docking and initiation of the SNARE complex (Figure 2) [69]. Munc18-2 deficient cells show defective degranulation and cytotoxicity [69].

1.4.5 Target cell death

Perforin is a pore-forming member of the membrane-attack-complex/perforin (MACPF) protein family [162, 163]. The structure of perforin share similarities with that of the complement factors in the membrane attack complex (MAC) [12]. Perforin is needed for the delivery of the pro-apoptotic granzymes into the target cell (Figure 2). However, the exact role of perforin is not clear. The C2 domain of perforin is required for Ca^{2+} -dependent membrane interaction, and subsequent oligomerization into pores made of 18-20 molecules, with an internal diameter large enough for the delivery of granzymes [163]. One hypothesis is that the granzymes directly enter the target cell through perforin pores [163]. Another hypothesis involves an uptake of perforin together with granzymes in endocytic vesicles, and a perforin-mediated disruption of these vesicles in the cytosol [163].

Granzyme B induces apoptosis in the target cell, whereas the role of other granzymes, including granzyme A, is less certain [164]. Granzyme A has been described to activate pro-inflammatory signals [165]. Apoptotic programmed cell death is fundamental for tissue homeostasis and occurs in a highly regulated series of events. In contrast to necrotic cell death, apoptosis typically does not induce inflammation. The series of events includes condensation of the nucleus and the cytoplasm, blebbing of the membrane, and generation of apoptotic bodies. The apoptotic bodies are rapidly recognized and ingested by phagocytic cells [166]. Apoptosis can be induced via different pathways; the intrinsic/mitochondrial pathway, the extrinsic/cell receptor pathway, or via granule exocytosis signals [166]. In the case of perforin-mediated lymphocyte cytotoxicity, granzyme B can directly induce apoptosis by cleavage of caspase 3, or indirectly via cleavage of Bid [167]. The truncated Bid translocates to the mitochondrial membrane and recruits Bax, which integrates with the mitochondrial membrane and induces release of cytochrome c from the mitochondria [168]. The

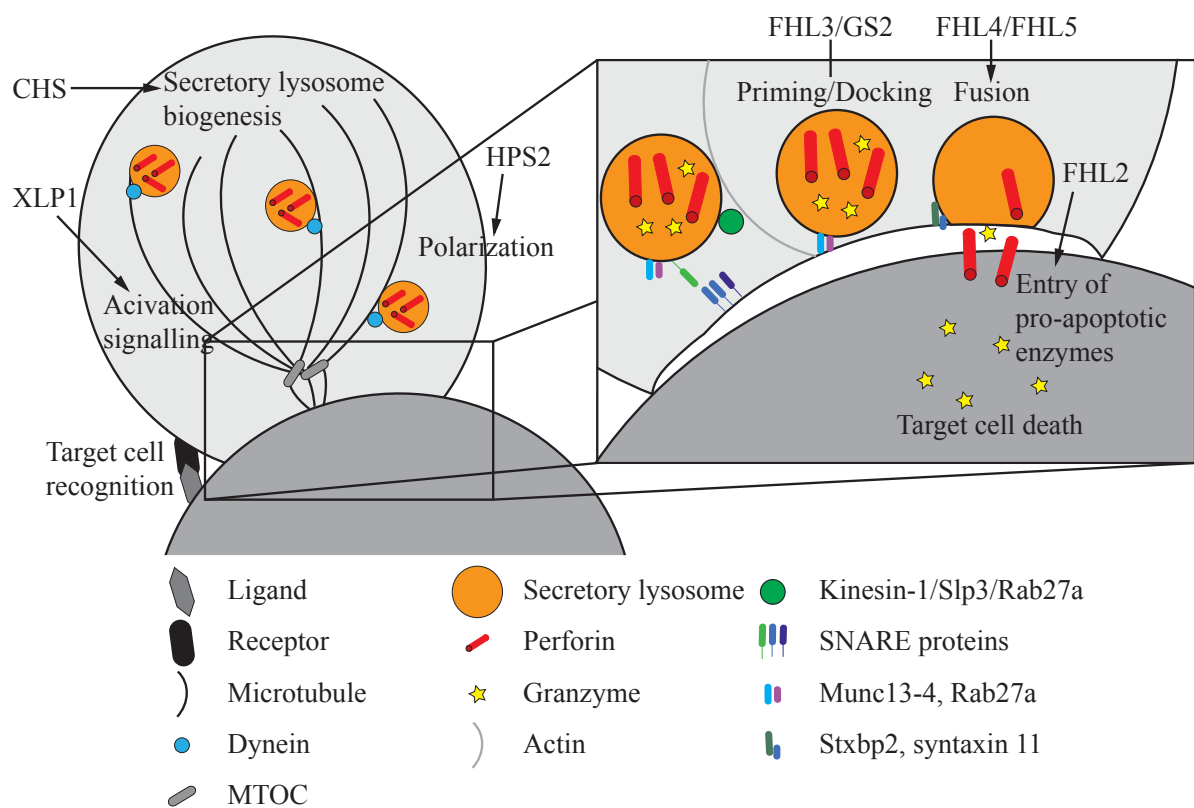
release of cytochrome c induces apoptosis via activation of caspase 9 and subsequently caspase 3 [169].

X-linked inhibitor of apoptosis (XIAP) is broadly expressed in hematopoietic cells [80]. XIAP belongs to the family of inhibitor of apoptosis, and is an inhibitor of caspase 3, 7, and 9 [170], and also involved in signaling pathways [171]. Patients with XIAP-deficiency suffer from XLP2, which is associated with development of HLH [80]. However, it is not clear how deficiency of XIAP causes disease. Cytotoxicity and degranulation is normal in XLP2 patients [80, 172].

Target cell killing via the extrinsic pathway involves engagement of the death receptors. In CTLs and NK cells directed exocytosis of the secretory granules delivers FasL to the cell surface [127]. The induction of FasL requires Ca^{2+} , but in contrast to the effector function of perforin, binding of FasL to its ligand is Ca^{2+} -independent [173]. Engagement of the death receptors Fas and TRAIL initiate recruitment of the adaptor protein Fas-associated death domain (FADD), which in turn recruits caspase 8 and 10. Active caspase 8 and 10 initiate the cascade that culminates in apoptotic cell death of the target cell [140].

Studies on in vitro cleavage of the caspase-3-like substrate aspartate-glutamate-valine-aspartate-7-amino-4-methyl-coumarin (DEVD-AMC) and proteolysis of the anti-apoptotic protein Bcl-2 revealed that immune cells derived from FHL patients are not inherently resistant to apoptosis induction [174]. Specifically, etoposide-induced and Fas-triggered activation of intracellular caspases appear to remain intact in FHL patients. However, the degree of spontaneous activation of caspase-3-like enzymes in activated lymphocytes was decreased in most patients studied [174]. These studies were the first to suggest components of the perforin-granzyme system as the underlying defect in FHL [174].

Figure 2. Schematic view of perforin-mediated lymphocyte cytotoxicity, depicting steps where the proteins defective in different forms of primary HLH are involved.



2 AIMS OF THE THESIS

The general aim of the thesis was to increase the clinical, genetic, and biological understanding of HLH and thereby improve diagnostics and survival for affected individuals. Furthermore, in a broader perspective, studies of the genetic and biological defects involved in lymphocyte cytotoxicity can gain insight into mechanisms of general significance for human biology.

The specific aims were:

Paper I:

- to provide an updated estimate of the annual incidence of primary HLH in Sweden between 1987 and 2006.
- to provide a minimal incidence of primary HLH, based on genetic and biological confirmed diagnoses, in Sweden between 2007-2011.
- to describe the clinical presentation and outcome in the affected individuals.

Paper II:

- to determine the frequency of *RAB27A* mutations in a cohort of patients with primary HLH not harboring mutations in the FHL-causing genes *PRF1*, *UNC13D*, and *STX11*.
- to describe the clinical presentation, including neurological involvement, in Griscelli syndrome type 2 patients.

Paper III:

- to describe how NK cell cytotoxicity and degranulation is affected by mutations in the genes encoding Munc13-4 and Rab27a.
- to study how engagement of different receptors regulate exocytosis of secretory lysosomes.

Paper IV:

- to expand the spectrum of *STXBP2* mutations in FHL5 patients.
- to describe the clinical presentation of FHL5 patients.
- to study NK cell function in FHL5 patients.

Paper V:

- to identify genetic defects in FHL patients without mutations in coding regions of *PRF1*, *UNC13D*, *STX11*, and *STXBP2*.

3 PATIENTS, MATERIAL, AND METHODS

This section will in brief describe the patients and methods included in **Paper I-V**. For details of the protocols, readers are referred to the method section in the individual papers.

3.1.1 Study populations and patient recruitment

In the first part of **Paper I**, the study population consisted of all children below the age of 15 years during the period 1987-2006 in Sweden, in average 1 599 272 per year. For identification of patients with primary HLH, all pediatric centers in Sweden were asked to report their patients with a possible diagnosis of HLH between 1987-2006. In addition, the Causes of Deaths and the Swedish National Inpatient registries, both held by the National Board of Health and Welfare in Sweden, were used to identify patients with a relevant diagnosis according to the International Classification of Diseases (ICD) between 1987-1996 (ICD-9; 202D, 202X, 277W) and 1997-2006 (ICD-10; D76.1, D76.2, D76.3, C96.1), respectively.

The patients included in the second part of **Paper I** and in **Papers II-V** have all been referred to us with a suspicion of HLH. The genetic studies started more than a decade ago, whereas functional studies of lymphocyte cytotoxicity have complemented and guided the genetic studies in the majority of patients since December 2005. After referral, sequence analyses of the known genes associated with FHL have been performed [175, 176, 177, unpublished data]. The patients were included in the different papers as specified below. In addition, healthy blood donors in Sweden have been used as controls.

In the second part of **Paper I**, all patients with genetically verified primary HLH and/or defective NK cell function consistent with primary HLH referred to us 2007-2011 were included.

In **Paper II**, 21 families, without identifiable mutations in the FHL-associated genes, *PRF1*, *UNC13D*, and *STX11*, were included. All patients fulfilled the HLH-2004 criteria for HLH [63], and had either undergone HSCT or had a survival of less than 1 year without HSCT. In the second part of **Paper II**, patients with a clinical diagnosis of GS2, from whom DNA was available, were included. In addition, we reviewed the medical literature of patients with *RAB27A* mutations.

In **Paper III**, patients with bi-allelic mutations *RAB27A* (n=3) and *UNC13D* (n=4), from whom fresh blood cells were available for functional studies of cytotoxic lymphocyte function, were included.

In **Paper IV**, patients without identifiable mutations in the FHL-associated genes, *PRF1*, *UNC13D*, and *STX11* were included, whereof eight families were identified with bi-allelic mutations in *STXBP2*.

In **Paper V**, Swedish infants with a known family history of HLH and/or defective NK cell cytotoxicity and/or degranulation between December 2005 and January 2011 were included. In addition, all patients from Sweden, Denmark, Norway, Finland, Slovenia, and Croatia with mutations in *UNC13D* identified at our unit were included in the study.

3.1.2 Statistical analyses

In **Paper I**, comparison between different categorical variables was performed by cross-tabulation using Pearson Chi-Square test, or alternatively the two-tailed Fisher's exact test when expected frequencies were small. SPSS statistics (version 20, IBM Corp., Armonk, NY) was used for the analyses. In **Paper III**, co-localization of proteins, assessed by confocal microscopy, was quantified as the Pearson coefficient *r*. Student's 2-tailed paired t-test was used for significance statistical analyses. Prism software (Version 5, Graph-Pad Software, La Jolla, CA) was used for analyses. In **Paper V**, statistical significance of results in the allele-specific quantitative real-time PCR was analyzed using the 2-tailed Mann-Whitney *U* test. SPSS statistics (version 20, IBM Corp.) was used for the analyses.

3.1.3 Mutation detection

Mutation detection by PCR and subsequent Sanger sequencing was performed in **Paper I-V**. First, genomic DNA was isolated from peripheral blood or, alternatively, from cultured fibroblasts according to standard procedures. Specific forward and reverse primers were designed for amplification of regions of interests by polymerase chain reactions (PCR), a method first described by Mullis *et al.* 1986 [178]. PCR facilitates selective, exponential amplification of DNA by denaturation of the double-stranded DNA, annealing of the specific primers, and elongation by a polymerase. This occurs during repeated temperature changes, using a thermal cycler. Amplified fragments were subsequently directly sequenced using chain-terminating inhibitors, based on the method described by Sanger *et al.* 1977 [179]. Fluorescently labeled dideoxy analogues of the normal deoxynucleoside triphosphates are randomly incorporated in the DNA strands and function as inhibitors of the DNA polymerase, resulting in differently sized sequences. The synthesized fluorescently labeled DNA fragments were subsequently separated by size, by capillary electrophoresis on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). SeqScape software (Applied Biosystems) was used for sequence analysis. BigDye terminator Version 3.1 Cycle Sequencing Kit (Applied Biosystems) was used for sequencing

3.1.4 Assessment of NK cell cytotoxicity

In **Papers II-V**, assessment of NK cell cytotoxicity was performed based on the 4 h ⁵¹Cr-release assay described by Schneider *et al.* [180]. First, peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by density gradient centrifugation. They were used as target cells, either freshly or activated by IL-2

(Proleukin, Novartis, Basel, Switzerland) incubation 36-60 hours. K562 cells (human erythroleukemia cell line, ATCC) were labeled with ^{51}Cr and used as target cells. Effector and target cells were co-incubated for 4 hours at different effector-to-target cell ratios. Lysis of the target cells result in a ^{51}Cr -release, which was measured using a gamma-counter. Lymphocyte cytotoxicity was calculated as lytic units (LU) at 25 % target cell lysis. A value of <10 LU was regarded as pathologically low. In **Paper II**, a value of 10-25 LU was defined as low.

3.1.5 Assessment of NK cell degranulation

Lamp-1, or CD107a, is a transmembrane protein in secretory lysosomes. Upon exocytosis of the secretory vesicles, the plasma membrane expression of CD107a is increased. This induction of CD107a expression was used to assess the exocytosis of secretory vesicles in **Papers II-V** [181]. Stimulation by K562 target cells was used for assessment of natural cytotoxicity, and stimulation by P815 (mouse mastocytoma cell line) cells coated with anti-CD16 was used to assess the ADCC. PBMCs were mixed with K562 cells or P815 cells supplemented with anti-CD16 antibody. Cells were incubated for 2 hours at 37°C in 5% CO₂, then spun down and resuspended in PBS supplemented with 2% fetal bovine serum (FBS) and 2 mM ethylenediaminetetraacetic (EDTA). Cells were stained with anti-CD3, anti-CD8, and anti-CD56 (all BD Bioscience, Franklin Lakes, NJ), and analyzed by flow cytometric analysis (FACSCalibur, BD Biosciences). Data were analyzed using FlowJo software (ThreeStar, Ashland, OR). Lymphocytes were gated on forward/side scatter. NK cells were gated on CD3⁻CD56⁺ lymphocytes, and induction of CD107a surface expression (ΔCD107a) was quantified.

For intracellular labeling, cells were surface-stained, fixed with 4% formaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS, permeabilized with PBS supplemented with 2% FBS, 2 mM EDTA, and 0.5% saponin (Sigma-Aldrich), and stained with fluorochrome-conjugated anti-CD63, anti-CD107a, anti-granzyme A, and anti-perforin mAbs.

3.1.6 Light and transmission electron microscopy of hair samples

Irregular accumulation of pigment in hair shafts has been described in GS2 patients. To evaluate this in **Paper II**, light microscopy and electron microscopy of hair from GS2 patients were performed. For light microscopy, small pieces of hair from the patients placed on a glass slide. The slides were mounted with Eukitt (O. Kindler GmbH & Co., Freiburg, Germany), and specimens were examined in an Eclipse C1000 microscope (Nikon, Tokyo, Japan). Images were obtained using a digital camera (DXM 1200F, Nikon) operating with ACT-1 software (Nikon). For transmission electron microscopy, small pieces of hair were fixed. Ultrathin sections of approximately 40–50 nm were cut and contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 10 (Philips, FEI, Eindhoven, the Netherlands) at 80kV. Digital images were taken with a MegaView III digital camera (Soft Imaging System GmbH, Münster, Germany).

3.1.7 Immunofluorescence and confocal microscopy

Confocal microscopy is an optical imaging technique, which compared to conventional microscopes allows for better optical resolution and reconstruction of three-dimensional structures. In **Paper III**, confocal microscopy was used to study fluorescently labeled proteins involved in NK cell cytotoxicity upon engagement of different receptors. Purified NK cells were stimulated with phorbol 12-myristate 13-acetate (PMA, Calbiochem, Darmstadt, Germany) and ionomycin (Sigma-Aldrich) and incubated together with S2 (*Drosophila* Schneider 2 cells) or K562 cells, or with ligand-coated beads on glass slides for 20 minutes at 37°C (Erie Scientific Company, Portsmouth, NH). In some experiments, cells were pre-treated with ML-9 (Calbiochem) for 20 minutes. For coating of beads, protein A-coated beads (Bangs Laboratories, Fishers, IN) were washed twice in water, incubated with purified human IgG (Sigma-Aldrich), or recombinant human ICAM-1-Fc (a gift from E. Long, National Institutes of Health, Rockville, MD) for 2 hours at 4°C, then washed and resuspended in RPMI 1640 medium. After stimulation, cells were fixed with 4% paraformaldehyde in PBS, and subsequently permeabilized. For permeabilization PBS supplemented with 0.5% saponin was used. Reactions were blocked in PBS containing 5% FBS, 0.1% BSA-c (Aurion, Wageningen, The Netherlands), and 2% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were mounted using Prolong Gold with 4,6-diamidino-2-phenylindole (Invitrogen, Paisley, UK), and images were acquired on a confocal microscope (DMIRE2, Leica, Solms, Germany) with a 63 × glycerol objective, using Leica Confocal Software (Version 2.61). ImageJ software was used for analyses (Version 1.410; Research Service Branch, National Institutes of Health, Bethesda, MD), with the Pearson correlation coefficient, r , determined using the JACOP plugin. Images for figures were generated by reconstruction of multiple optical sections.

3.1.8 RNA extraction, cDNA synthesis and amplification, and analyses

In **Paper V**, the *UNC13D* transcript was analyzed to identify a possible defect in splicing of the mRNA. Total RNA was extracted from white blood cells (RNeasy, QIAGEN, Hilden, Germany), and cDNA was synthesized with oligo(dT)₂₀ primed reverse transcription (SuperScript III, Invitrogen) according to the manufacturer's protocol. Overlapping cDNA fragments of *UNC13D* were amplified using 9 different specific primer pairs. For 3'-rapid amplification of cDNA ends (RACE) PCR, cDNA was synthesized using an oligo(dT)₂₀ primer marked with an M13R-tag. This allowed sequencing of an unknown end of the transcript. Amplified products were separated by agarose gel electrophoresis, extracted (QIAquick gel extraction kit, QIAGEN), and cloned (TOPO-TA cloning kit, Invitrogen). Plasmid DNA was isolated (GeneJET plasmid miniprep kit, Fermentas, Glen Burnie, MD) and subsequently sequenced as described above.

3.1.9 Allele-specific quantitative RT-PCR

For evaluation of a possible defect in the *UNC13D* transcript levels, allele-specific quantitative real-time PCR was performed in total white blood cells and in specific sub-populations in **Paper V**. PBMCs were isolated by density gradient centrifugation, and specific cell populations were consecutively isolated by magnetic positive selection using anti-CD14, anti-CD4, anti-CD8, and anti-CD56 mAb-coated beads, respectively (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the isolated cell populations was assessed by flow cytometry using fluorochrome-conjugated mAbs against CD3 (eBioscience, San Diego, CA), CD4 (Invitrogen), CD8 (Invitrogen), CD14 (BD Bioscience), CD45 (BD Bioscience), and CD56 (BD Bioscience).

RNA was isolated using TRIzol (Invitrogen) according to manufacture's instructions, and cDNA was synthesized as described above. Two different reverse primers were designed for discrimination of a single nucleotide polymorphism, c.888G>C, located in exon 11 in *UNC13D*. 100 ng cDNA was mixed with 10 µL of Power SYBR Green PCR master mix (Applied Biosystems) and 10 pmol of forward and reverse primers in a total volume of 20 µL. The reactions were analyzed by real-time PCR (ABI 7900 HT, Applied Biosystems) using the standard curve method and analyzed with SDS software (Version 2.2.1, Applied Biosystems).

3.1.10 Western blot analysis of Munc13-4 expression

In **Paper V**, the genetic findings were confirmed with Western blot analyses. PBMCs were lysed in lysis buffer (20mM Tris, pH 7.4, 1mM EDTA, 1% Triton X-100, 150mM NaCl) with protease inhibitors (Roche Diagnostics, Basel, Switzerland), disrupted and centrifuged for 15 minutes at 14 000g. Protein content was determined by the Bradford assay (Bio-Rad, Hercules, CA). Protein was loaded and analyzed using SDS-PAGE separation and Western blotting (NuPAGE, Invitrogen). The rabbit polyclonal antibody to Munc13-4 (Protein Technologies Group, Chicago, IL), raised against amino acids 1-236) and ERK1 (Santa Cruz Biotechnology, Santa Cruz, CA) were used for Western blotting.

3.1.11 Genotyping of microsatellite markers

For evaluation of haplotypes around the *UNC13D* locus, genotyping with microsatellite was performed in **Paper V**. Fluorescently labeled primers were used in PCR according to standard procedures. PCR products were separated by capillary electrophoresis on an ABI 3730 DNA Analyzer (Applied Biosystems) with GeneScan 400HD Rox (Applied Biosystems) as size standard and analyzed with Peak Scanner Version 1.0 software (Applied Biosystems).

4 RESULTS AND DISCUSSION

4.1 EPIDEMIOLOGY

The first incidence study of FHL was published in 1991 by Henter *et al.* [104]. In that study, an annual incidence of 1.2 per million children below the age of 15 years in Sweden, corresponding to around 1 per 50 000 live born children, was reported [104]. Sweden is a suitable country for incidence studies attributable to the well-organized personal identification numbers and a structured health care system with national registries. The population studied by Henter *et al.* consisted of all children below the age of 15 years in Sweden between the years 1971 and 1986. For identification of patients, all pediatric centers in Sweden were contacted and asked to report their patients with FHL and disorders resembling FHL. Furthermore, the causes of death according to death certificates of all children (n=19 542 children) deceased during the study period were reviewed. Patients with previous immunosuppressive therapy, neoplastic cells, and Langerhans-cell histiocytosis were excluded from the study. In total, 32 patients were identified, of which 19 of 32 patients (66 %) were diagnosed with FHL post mortem.

A Japanese incidence study reported the annual incidence of HLH to be 1.25 per million individuals in Japan [182]. Questionnaires were sent to 272 institutions with pediatric and adult hematologists in Japan, asking them to report patients with HLH, with a response rate of 74 %. In contrast to the study by Henter *et al.*, the Japanese study included both patients with primary and secondary HLH. 3.5 % of the patients were classified as having FHL based on a familial history, a genetic diagnosis, an age <2 years, impaired NK cell function, or CNS involvement [182]. Altogether, 56.5 % of the patients reported were below the age of 15 years [182]. Infection-related HLH represented approximately half of the total number of cases, of which the majority was secondary to EBV-infections [182]. EBV-associated HLH is especially common in the Asian population [87]. Malignancy-associated HLH represented 23.3 % of the patients and 9.3 % were secondary to autoimmune diseases [182].

Since 1991, when the first incidence study in Sweden was published, the clinical and biological understanding of HLH has increased. Moreover, diagnostic guidelines are now available facilitating the diagnostics. We, therefore, in **Paper I**, hypothesized that the true incidence of primary HLH could be higher than previously estimated. Two different strategies were used for patient identification. First, written inquiries were sent to all pediatric centers in Sweden, asking them to report their patients with HLH between 1987 and 2006. Secondly, data from the registries of the Swedish National Board of Health and Welfare were reviewed. Patients between 0 and 15 years of age were considered for inclusion, while those with an alternative diagnosis or with a malignant diagnosis at the time of diagnosis of HLH, suggesting secondary malignancy-associated HLH, were excluded from the study. Furthermore, patients with a relapse-free survival of more than one year off treatment were excluded from the study, regarded as most likely having secondary HLH. Medical files from 77 patients with possible HLH were requested from the treating medical centers. Of these 77

patients, 31 fulfilled at least five of the eight criteria for HLH according to HLH-2004 (1987-1996: n=15, 1997-2006: n=16). In addition, six patients with missing values for at least three criteria but that fulfilled at least four of the criteria (1987-1996: n=6, 1997-2006: n=0), and one patient with a known familial history (1987-1996: n=1, 1997-2006: n=0), were still regarded as having primary HLH.

Thus, in total 38 patients were regarded as having primary HLH in Sweden between 1987 and 2006, giving an estimated annual incidence of 1.2 per million children aged less than 15 years, corresponding to 1.8 patients per 100 000 live born children. Hence, the estimated annual incidence was unchanged compared to what was reported by Henter *et al.* in 1991 [104]. Similarly, no major difference was seen in the annual incidence of primary HLH among the cohort of patients aged less than one year, eleven (1987-2006) compared to ten (1971-1986) per million children, respectively. Of note, the annual incidence in the northern part of Sweden (“Norrland”) was higher as compared to other parts of Sweden (“Svealand” and “Götaland”), 2.1 compared to 1.0 and 1.1 per million children below the age of 15 years. A founder effect of an aberration causative of FHL, that will be discussed later on, in the northern part of Sweden (“Norrland”) together with a stronger inbreeding in this area [183], might contribute to the higher annual incidence. The second part of **Paper I**, aimed to provide a minimal incidence of primary HLH based on defective cytotoxic lymphocyte function and/or defective NK cell degranulation, or a verified genetic defect indicative of primary HLH. During the subsequent five-years period (2007-2011) twelve such patients were identified, giving an annual incidence of 1.5 per million children below the age of 15 years, corresponding to 2.2 patients per 100 000 live born children.

Even though the awareness of HLH has increased and diagnostic guidelines are available, there are still difficulties in the diagnostics of HLH. The clinical presentation can vary markedly between different patients and some patients may still die before they are correctly diagnosed. In **Paper I**, patients with non-detected HLH are obviously not included in the study. In contrast, the study by Henter *et al.* included also patients identified through thorough review of all childhood deaths. This could contribute to an underestimation of the estimated incidence in **Paper I**. Moreover, in an attempt to exclude patients regarded as having secondary HLH, patients over the age of 15 years, patients with a disease free survival > 1 year without treatment, and patients with a malignant diagnosis at the time of HLH were excluded from the study. It is now known that patients with genetically verified primary HLH can present in adolescence or later [184, 185], **Paper IV**. Furthermore, patients with hypomorphic mutations in the genes associated with primary HLH may present hematological malignancies [186]. This can also result in an underestimation of primary HLH in our study.

SCID has previously been reported with an estimated minimal incidence of 1 per 100 000 live births in France [187] and 1.4 per 100 000 live born children in a Swedish study [188]. Of note, a higher SCID incidence, 2.6 per 100 000 full-term neonates, was reported in a prospective population-based study using an assay enumerating the number of T cell receptor excision circle. A similar prospective screening of neonates, if feasible, would likely also reveal a higher true incidence of primary HLH.

4.2 GENETICS OF HLH

4.2.1 Background

When the projects included in this thesis were initiated 2007 three genes with FHL-causing mutations were known; *PRF1*, *UNC13D*, and *STX11* [66-68]. Their individual contribution to FHL varies in different populations [189, 190]. In a study by zur Stadt *et al.*, 40 % of their FHL patients did not harbor mutations in *PRF1*, *UNC13D*, and *STX11*, ranging from 19 % in patients of Turkish origin to 70 % in patients of German origin [190]. In a study by Horne *et al.*, 25 of 49 patients (51 %) of the families analyzed for mutations in *PRF1*, *UNC13D*, and *STX11* were reported to be without a molecular diagnosis [189]. In patients from the Nordic countries, the proportion of patients without a molecular diagnosis was significantly higher (13 of 14 patients; 93 %) compared to that observed in patients from Turkey (ten of 24 patients; 42 %) and the Middle East (two of eleven patients; 18 %) [189]. The high proportion of patients without a molecular diagnosis, especially in the Nordic countries, has been problematic in regards to diagnostics and carrier testing as well as for the possibility of prenatal diagnostics and preimplantation genetic diagnosis (PGD).

4.2.2 *RAB27A* mutations

As described in the introduction, GS2 is a primary immunodeficiency associated with development of HLH that is caused by mutations in *RAB27A* [72]. *RAB27A* encodes the 221 amino acids protein Rab27a, a member of the small GTPase family involved in vesicular fusion and trafficking [72]. In addition to its role in cytotoxic lymphocytes, Rab27a, together with melanophilin and myosin Va, is required for the transport of the pigment-containing melanosomes [191]. Consequently, GS2 patients display a partial albinism in addition to the immunological defect [72]. In **Paper II**, we hypothesized that some of the patients diagnosed as having FHL instead could be affected by GS2, especially in the Nordic countries where many individuals normally are fair-haired. Accordingly, we identified heterozygous compound mutations in one Swedish family out of 21 families, which had been tested and found negative for mutations in *PRF1*, *UNC13D*, and *STX11*. This family included three affected individuals whereof two were identical twin-sisters, all previously diagnosed as having FHL. One of the identified mutations was novel, a substitution of the last nucleotide in exon 3, c.239G>C. This mutation is predicted to interfere with mRNA splicing by disrupting the consensus splice donor site [192]. The other mutation was a nonsense mutation, c.550C>T, introducing a premature stop codon, p.Arg184X, previously described by Menasche *et al.* [72]. None of the mutations were identified among 96 healthy blood donors.

In the second part of **Paper II**, three patients with a clinical diagnosis of GS2 were analyzed for mutations in *RAB27A*. One patient of Danish origin, previously described by Trottestam *et al.* [113], harbored the above-mentioned p.Arg184X mutation in a homozygous state. The second patient, of Pakistani origin, was identified with a homozygous splice acceptor site mutation, c.240-2A>C, predicted to disrupt the mRNA splicing. This novel mutation was not identified among 96 healthy blood donors. The

third patient, of German origin, carried a homozygous mutation, c.148_149delinsC, previously described by Mamishi *et al.* [193], resulting in a frameshift and a premature stop codon, p.Arg50GlnFsX35.

The identification of *RAB27A* mutations in three affected individuals from one family diagnosed with FHL highlights the importance of considering GS2 among patients with HLH, especially in fair-haired individuals as the partial albinism easily can be overlooked. Further strengthening this, two of the three patients included in part two of **Paper II** were first diagnosed as having FHL and only later correctly diagnosed as having GS2.

4.2.3 *STXBP2* mutations

In 2009 two groups independently described *STXBP2* as the fourth gene associated with FHL, more specifically with FHL5 [69, 70]. *STXBP2* encodes the 593 amino acids protein Munc18-2. In the paper by zur Stadt *et al.* 13 patients of German, Turkish, Saudi Arabian, and Czech origin were described [70]. Together they harbored five different missense mutations, one 3 bp deletion, two frameshift deletions, and one splice acceptor site mutation of exon 15 [70]. The splice site mutation, c.1247-1G>C, frequently resulted in a deletion of exon 15 and a frameshift, introducing a stop codon, p.Val417LeuFsX126 [70]. In addition, other splice products were observed. Interestingly, all patients who developed HLH after the age of one year carried this specific splice site mutation on at least one of the alleles [70].

In the paper by Côte *et al.*, nine individuals of Saudi Arabian, Palestinian, and Turkish origin were described with mutations in *STXBP2* [40]. Five patients from three families, all of Saudi Arabian origin, carried a homozygous missense mutation, p.Pro477Leu, whereas the remaining patients carried the above described splice site mutation, c.1247-1G>C [40]. The splice mutation was described to result in an in-frame exchange of the 17 first codons in exon 15 by 19 codons from the intronic sequence [40].

In **Paper IV**, we describe eleven patients from eight unrelated families diagnosed with FHL5. Nine of the patients carried bi-allelic mutations in *STXBP2*. In addition, two siblings, from whom DNA was not available for genetic analyses, were included. Four different missense mutations, one nonsense mutation, one out-of-frame deletion, two in-frame deletions, and one splice acceptor site mutation were identified among these patients. The splice site mutation, previously described by zur Stadt *et al.* [70] and Côte *et al.* [40], was identified in four of eight families of Caucasian or Asian origin. Altogether 43 % of the families reported by zur Stadt *et al.*, Côte *et al.*, and in **Paper IV**, carried this particular splice site mutation. In addition to the splice site mutation, one of the missense mutations, p.R405W had previously been described by zur Stadt *et al.* [70] while all the other mutations identified in **Paper V** were novel. None of the novel non-truncating mutations were identified among 95 healthy blood donors.

In a subsequent paper by Cetica *et al.* four patients were described with four different missense mutations in *STXBP2*, whereof one was novel, p.Glu132Ala [194]. The so far

largest cohort of FHL5 patients was reported in 2012 by Pagel *et al.*, where in total 37 patients, from 28 families, with bi-allelic *STXBP2* mutations were described [195]. Sixteen of the patients had previously been reported with *STXBP2* mutation [70, 184].

4.2.4 *UNC13D* mutations

UNC13D was described as causative of FHL3 in 2003 [67]. Since then several papers describing the spectrum of mutations in *UNC13D* has been published [176, 189, 190, 196-200]. A European collaborative effort to describe the genotype-phenotype in FHL3 patients resulted in report of 84 FHL3 patients in 2011 [201].

Altogether seven patients, from six unrelated families, in our patient cohort were identified with only mono-allelic mutations in *UNC13D*. This prompted us to search for mutations outside the coding regions of the gene in **Paper V**. A phylogenetic hidden Markov model based on sequences from 17 vertebrates was used for identification of evolutionary highly conserved regions [202].

By this strategy, four highly conserved non-coding regions were identified. By sequence analysis, a variant, c.118-308C>T, was identified in one of the conserved intronic regions in five of the seven patients with mono-allelic *UNC13D* mutations but not in 96 healthy controls. This variant, in intron 1, had previously been described in a heterozygous state in one single patient diagnosed with FHL3 [196]. Two additional variants were identified in intron 1, constituting a common polymorphism c.117+59C>T (rs3744010) and a variant c.118-176G>C. The latter variant was identified in a homozygous state in three and in a heterozygous state in twelve of 96 healthy blood donors, thus unlikely disease-causing. When sequencing other FHL patients without a known molecular diagnosis, two additional patients were identified with the same mutation, c.118-308C>T, in a heterozygous state, and, importantly, one patient was identified with this mutation in a homozygous state. Western blot analysis of whole cell lysates prepared from PMBCs from patients with the c.118-308C>T mutation did not show Munc13-4 protein.

To elucidate whether the c.118-308C>T mutation had an impact on mRNA splicing, as previously suggested by Santoro *et al.* [196], or on the regulation of *UNC13D* transcription, the transcript was analyzed in patients and in heterozygous carriers of the mutation. Nine overlapping fragments of the *UNC13D* transcript were amplified in the patient homozygous for the c.118-308C>T mutation. Similarly as in the control, all fragments were readily amplified in the patient and subsequent sequencing revealed correctly spliced products indicating that the variant did not interfere with splicing. Next, allele-specific quantitative real time PCR was performed in heterozygous carriers of the c.118-308C>T mutation, which were also heterozygous for a linked heterozygous polymorphism in exon 11. Healthy individuals, carrying the polymorphism in a heterozygous state but not the c.118-308C>T mutation were used as controls. Analysis of transcription in white blood cells (WBCs) revealed a significantly diminished transcription of the allele with the polymorphism in heterozygous carrier of the intron 1 mutation representing only 27 % of the total transcripts compared to 50 % in the controls ($p<0.05$). For further evaluation of a possible role of the mutation in the

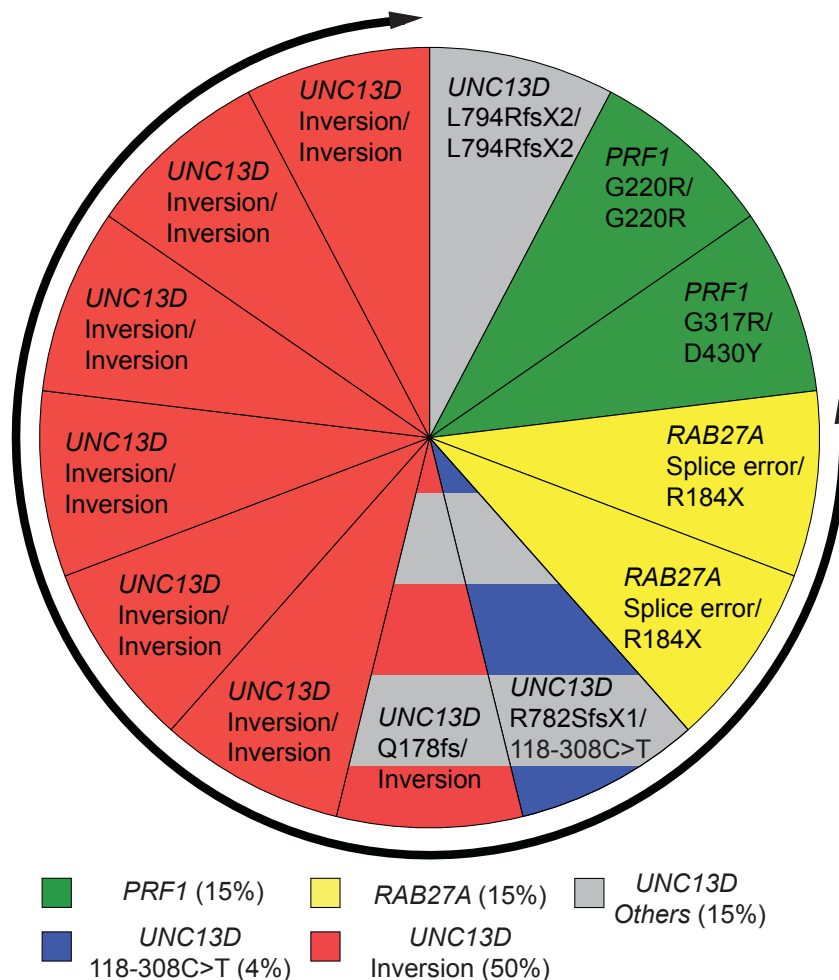
regulation of transcription, sequentially isolated CD14⁺, CD4⁺, CD8⁺, and CD56⁺ cells were used for allele-specific transcription as described above. Notably, the allele harboring the c.118-308C>T mutation represented less than 10 % of the transcript in CD4⁺, CD8⁺, and CD56⁺ cells. This was significantly less than what was observed in CD14⁺ cells. In the controls, the frequency of transcripts from the two different alleles was similar. These findings suggested an impact of the c.118-308C>T mutation specifically in lymphocytes. The sequence around the position of the intron 1 mutation displays homology to the Ets-1 and STAT4 consensus binding sites, which are disrupted by the mutation. This suggests that the sequence could represent an intronic enhancer of transcription. Of note, Ets-1 and STAT4 are both highly expressed in cytotoxic lymphocytes [203, 204]. Microsatellite analyses of carriers of the intron 1 mutation indicated a common haplotype around the *UNC13D* locus, suggesting a founder effect. The intron 1 mutation has, in our laboratory, been identified in patients spread across Europe.

The c.118-308C>T mutation was not identified in two of the original seven patients with mono-allelic *UNC13D* mutations. Importantly, two new patients were identified with monoallelic mutations harboring the c.118-308C>T in a heterozygous state. This suggested the presence of another non-coding aberration in *UNC13D*. Examination of SNPs located in exonic and intronic regions of *UNC13D* revealed a common haplotype on the second allele in patients with mono-allelic *UNC13D* mutations. Interestingly, other FHL patients, without a known molecular diagnosis, shared this second haplotype in a homozygous state. Nine overlapping fragments of the *UNC13D* transcript were amplified in three patients homozygous for this haplotype. In contrast to what was seen in healthy controls, the last two fragments were not amplified in the patients, suggesting an aberration affecting the 3' end of the transcript. 3'-rapid amplification of cDNA ends PCR was performed to obtain the 3' end sequence of the transcript. Cloning and sequencing of the PCR products revealed a correct sequence up to the end of exon 30, followed by a sequence blasted to a region 253 kb downstream of the gene using BLAT [205]. This suggested an inversion of 253 kb straddling the *UNC13D* locus. Sequence analysis mapped down the breakpoints to two 25 bp-fragments of Alu elements, identical in sequence. Similarly, genomic rearrangement by paring of inverted Alu repeats has been described in X-linked recessive hemophilia A and Alport syndrome [206, 207]. However, to the best of our knowledge this represents the first paracentric inversion alone responsible for an autosomal recessive disease in humans. A multiplex PCR assay was developed for easy detection of the inversion. One of 190 healthy blood donors was found to carry the inversion in a heterozygous state. Western blot analysis of whole cell lysates prepared from PMBCs from patients with the inversion in a homozygous state did not show any Munc13-4 protein. Microsatellite markers indicated a common haplotype around the *UNC13D* locus in carriers of the 253 kb inversion, all of Scandinavian origin. A geographic gradient was observed with a high frequency of the inversion in the northern part of Sweden, suggesting a founder effect.

4.2.5 Mutation spectrum of primary HLH

Between December 2005 and January 2011, 13 Swedish patients with an age at onset below three years and that fulfilled the diagnostic criteria for HLH including defective NK cell cytotoxicity came to our attention (Figure 3). Of these, two patients carried bi-allelic *PRF1* mutations. One infant, of Afghani origin, harbored a novel *PRF1* mutation, p.Gly220Arg, whereas the other infant, of Swedish origin, harbored heterozygous compound mutations, p.Glu317Arg and p.Asp430Tyr, in *PRF1*. The latter patient has previously been described by Bryceson *et al.* [161]. One patient, of Somali origin, carried a novel homozygous mutation in *UNC13D*, p.Leu794ArgfsX2. These three patients are mentioned in **Paper V**. Importantly, the genetic aberrations identified in **Paper II** and **Paper V** could provide a molecular diagnosis for the remaining ten patients (77 %, Figure 3). Likewise, Côte *et al.* reported that the great majority of the FHL patients (90 %) in their cohort now have a known genetic diagnosis [69].

Figure 3. Mutated alleles in children aged less than three years that fulfilled the diagnostic criteria for HLH, including defective NK cell cytotoxicity in Sweden between December 2005 and January 2011.



Patients provided a molecular diagnosis in studies included in this thesis are marked with a black arrow. Adapted from **Paper V**.

4.3 LYMPHOCYTE CYTOTOXIC FUNCTION IN PRIMARY HLH

4.3.1 NK cell function in Griscelli syndrome type 2

In **Paper II**, NK cell activity was evaluated in six patients carrying bi-allelic *RAB27A* mutations diagnosed as having GS2. One patient displayed an NK cell activity of 22 LU, thus above the limit set as pathological. However, upon repeated analysis after treatment this had decreased to 1 LU. Similarly, two other patients had an NK cell activity above the limit set as pathological, 20 LU and 11 LU, respectively. NK cell activity in the remaining three patients was below 10 LU. Thus, only half of the patients initially had a defective NK cell function consistent with the diagnostic criteria for HLH [63].

In **Paper III**, analyses of NK cell function in GS2 patients as well as FHL3 patients with bi-allelic *UNC13D* mutations were performed. The three GS2 patients included in this paper were also included in **Paper II**. NK cell activity in all FHL3 patients was less than 10 LU. In 2007, Gazit *et al.* reported natural cytotoxicity but not CD16-mediated ADCC to be defective in a patient with a homozygous *RAB27A* mutation [208]. To evaluate this in our cohort of patients, degranulation was assessed using the CD107a assay. In contrast to what was seen in healthy adult and infant controls, degranulation in the GS2 patients, as well as the FHL3 patients, was defective (< 3 %) after incubation with K562 cells. Similarly, anti-CD16 mAb stimulation induced degranulation in the controls, whereas degranulation was defective or impaired in the GS2 and FHL3 patients. Thus, our data suggest that degranulation induced by receptors for natural cytotoxicity or ADCC are Rab27a-dependent. The defects in cytotoxicity and degranulation were less profound in the GS2 patients compared to the complete loss of function in NK cell function seen in the FHL3 patients. This small residual cytotoxicity and degranulation is consistent with a later onset of HLH and longer periods of disease-free remission sometimes seen in GS2 patients [209]. Activation of the effector cells with IL-2 for 72 hours restored cytotoxicity and degranulation to a greater extent in GS2 patients compared to what was seen in the FHL3 patients.

4.3.2 Function of Rab27a and Munc13-4 in exocytosis

Munc13-4 has previously been shown to interact with GTP-bound Rab27a [210, 211]. However, the exact role of these proteins in the exocytosis of secretory granules is unclear. In **Paper III**, the localization of these proteins in relation to perforin in NK cells was assessed using confocal microscopy. In resting cells, Rab27a and Munc13-4 did not display a general overlap with perforin. Upon activation of NK cells with PMA and ionomycin, perforin was clustered around a spot of α -tubulin labeling, at the center of microtubule network, likely reflecting the migration of the secretory lysosomes towards the microtubule organizing center. PMA and ionomycin stimulation significantly induced the colocalization of Rab27a with perforin, as well as the colocalization of Munc13-4 with perforin. Similarly, increased colocalization of Rab27a and Munc13-4 with perforin was observed after incubation with K562 in cells forming conjugates with the target cells. This corroborated findings previously reported in CTLs [212].

ML9, an inhibitor of myosin light chain kinase, was used to study whether the recruitment of Rab27a and Munc13-4 was myosin-dependent or not. The use of ML-9 prohibited degranulation, and inhibited the colocalization of perforin with Rab27a and Munc13-4, respectively, in a dose-dependent manner. Thus the recruitment of Rab27a and Munc13-4 are myosin-dependent events. The activation-induced myosin-dependent colocalization of Rab27a and Munc13-4 with perforin suggests recruitment of these proteins from heterotypic vesicular compartments. In 2007 Menager *et al.* described a concept of lytic granule maturation requiring cooperation of endosome-derived exocytic vesicles and lysosome-related, perforin-containing vesicles in CTLs [212]. That study was performed using overexpression of fluorescently labeled membrane-fusion proteins that together with chronic cytokine activation might alter cellular morphology and function. Thus, our findings that engagement of activation receptors induces endogenous Rab27a and Munc13-4 colocalization with perforin in freshly isolated NK cells provide support for some of their conclusions.

Munc13-4 has previously been described as necessary for the recruitment of Rab27a in CTLs [212]. In **Paper III**, recruitment of Rab27a similarly failed in Munc13-4 deficient NK cells. In our study of Rab27a-deficient cells, perforin was clustered at one point of the cells upon stimulation with PMA and ionomycin. However, Munc13-4 and perforin colocalization did not increase. These results suggest a reciprocal dependency of the presence of Rab27a and Munc13-4 for degranulation.

As the next step, recruitment of Rab27a and Munc13-4 to the perforin-containing granules by engagement of individual receptors was investigated. As mentioned in the introduction, engagement of LFA-1 has been shown to induce polarization of the secretory lysosomes but not degranulation, whereas engagement of CD16 potently induces degranulation but not polarization [137]. In **Paper III**, engagement of LFA-1 by ICAM-1-coated beads was shown to significantly induce colocalization of Rab27a, but not Munc13-4, with perforin. Conversely, engagement of CD16 by IgG significantly induced colocalization of Munc13-4 but not Rab27a.

In the next part of the study, *Drosophila* S2 cells, transfected with ICAM-1, CD48, or ULBP1, were used as target cells [213]. CD48 and ULBP1 are the ligands for the NK cell receptors 2B4 and NKG2D, respectively. For assessment of CD16-signalling, S2 cells were incubated with human serum containing antibodies to oligosaccharide moieties expressed by the S2 cells [214]. The use of S2-ICAM-1 and serum-precoated S2 cells, respectively, corroborated the previous findings using the ICAM-1 and IgG-coated beads. NK cells conjugated to S2-CD48 or S2-ULBP1, alternatively, significantly induced colocalization of Rab27a, but not Munc13-4, with perforin. Conjugation with S2 cells coexpressing CD48 and ULBP1 induced colocalization of both Rab27a and Munc13-4 with perforin, and S2 cells coexpressing ICAM-1, CD48, and ULBP1 resulted in the greatest increase of colocalization. These data suggest that recruitment of Rab27a and Munc13-4 to perforin-containing granules is directed by qualitatively different signals from distinct receptors. For recruitment of Munc13-4, engagement of receptor(s) inducing high Ca^{2+} mobilization and degranulation was needed (CD16 or NKG2D and 2B4 synergy) in contrast to Rab27a that was recruited upon engagement of receptors not inducing strong Ca^{2+} influx or degranulation.

Ligands for the receptors that recruit Rab27a are in most physiological conditions more widely expressed than those needed for Munc13-4, indicating that signals for Rab27a recruitment might proceed those of Munc13-4 recruitment.

4.3.3 NK cell function in FHL5

In **Paper IV**, NK cell function was evaluated in nine of the eleven FHL5 patients described. NK cell function was pathologically low (<10 LU) in all patients tested. Furthermore, NK cell degranulation was assessed in eight of the patients. Degranulation in response to K562 cells was less than 2 % in all patients. Notably, after IL-2 stimulation for 48-72 hours, K562 cell lysis and degranulation increased considerably, similarly to what was seen in FHL4 patients harboring *STX11* mutations [161]. These results are consistent with the findings reported by zur Stadt *et al.* and Côte *et al.* [69, 70].

4.3.4 NK cell function in different subgroups of FHL3

FHL3 patients were first described with defective NK cell cytotoxicity and degranulation by Feldman *et al.* in 2003 [67]. Other papers have later confirmed the functional defects in FHL3 patients [176, 215] In **Paper V**, two novel genetic aberrations causative of FHL3 are described, a intron 1 mutation, c.118-308C>T, and a 253 kb inversion straddling the *UNC13D* locus. NK cell function was evaluated and found defective in most patients with these aberrations. In patients harboring the intron 1 mutation on at least one allele, NK cell-mediated lysis, determined as lytic units, was <10 LU (the limit typically regarded pathological) in all patients but one (11 LU). Similarly, of the FHL3 patients with at least one allele of the 253 kb inversion, all displayed pathologic NK cell cytotoxicity (<10 LU) except for one patient (13 LU). Importantly, all patients but two displayed normal or close to normal NK cell frequencies.

Furthermore, degranulation in these groups of FHL3 patients, and in addition patients harboring other bi-allelic *UNC13D* mutation, was evaluated. Healthy controls and FHL2 patients were used as controls. In response to K562 cells, all patients with at least one allele carrying the intron 1 mutation displayed defective degranulation in opposite to the healthy adult and infant controls as well as the FHL2 patients. Among patients with at least one allele with the 253 kb inversion, three displayed degranulation ≥ 3 % (the limit set as pathological) during flares of HLH. Upon repeated analysis, degranulation was pathological in all patients. Of interest, degranulation by IL-2 activated cells was significantly more increased in patients carrying the inversion on at least one allele as compared to patients with the intron 1 mutation and other *UNC13D* mutations. Likewise, cytotoxicity in IL-2 activated NK cells was higher in patients carrying allele(s) with the inversion relative to patients harboring the intron 1 mutation or other *UNC13D* mutations. Such increase of NK cell function in IL-2 activated cells have previously been observed in patients with missense mutations in *UNC13D*, as well as in FHL4 and FHL5 patients [69, 70, 161, 176, 184, 216].

4.3.5 Functional studies of primary HLH

In summary, assessment of lymphocyte cytotoxicity has become important in the diagnostics of primary HLH and is of great value for guidance of genetic analyses, which often are time-consuming and expensive. As described above, pathological NK cell cytotoxicity is observed in patients with inherited defects in the perforin-mediated cytotoxic pathway. However, a decreased NK cell function can be observed also in patients with reduced frequencies of circulating cytotoxic cells, as is often seen in patients with secondary HLH. Thus, assessment of NK cell cytotoxicity is not always sufficient for distinguishing between primary and secondary forms of HLH [217]. Therefore, other complementary methods are of value in the diagnostics of patients with suspected HLH.

The use of degranulation assays in the diagnostics of primary HLH was recently evaluated in 494 patients with suspected HLH [217]. Quantification of NK cell degranulation using the Δ CD107a assay with a 5 % cut-off provided a 96 % sensitivity and 88 % specificity for genetic degranulation disorders [217]. Active disease or immunosuppression did not affect results. In patients with secondary HLH, 98 % (n=59) displayed normal resting NK cell degranulation [217]. Assessment of NK cell degranulation was thus suggested useful in the differentiation between primary and secondary HLH [217].

FHL2 patients display a defective cytotoxic lymphocyte function whereas the degranulation is functioning [66, 161]. Expression of perforin assessed by flow cytometry is reduced in the great majority of FHL2 patients, and thus another important tool in the diagnosis of FHL2 patients [217]. FHL3, FHL4, and FHL5 patients all display both a defective cytotoxic function and defective degranulation [69, 70, 161]. In cells from FHL4 and FHL5 patients, a partial pick up of function after stimulation with IL-2 has been described [69, 70, 161]. Even though not typically seen in FHL3, a similar pick up of function in activated cells has been seen in some FHL3-patients such as the patients carrying the 253 kb inversion [176, 184, 217].

Among other immunodeficiencies associated with development of HLH, cells from GS2 patients also display reduced NK cell cytotoxicity and degranulation [**Paper II**, **Paper III**, 186]. Similarly, NK cell function and degranulation have been described as deficient in patients with CHS [217, 218]. CTL cytotoxicity, assessed by a lysis assay on anti-CD3 labeled L1210 target cells, was absent in CHS patients with early onset HLH, whereas the CTL cytotoxicity was in the low normal range in patients who did not develop HLH [218]. Based on these data, CTL cytotoxicity was suggested predictive for the risk of development of HLH in CHS patients [187]. In CTLs from an HPS2 patient carrying bi-allelic *AP3B1* mutations, cytotoxicity and degranulation was impaired [75, 131]. Of note, the base-line expression of CD107a in resting cells was reported higher than in the controls [75, 131].

In XLP1 patients, 2B4-mediated NK cell cytotoxicity is defective, whereas CD2 and CD16 induced cytotoxicity is normal [139]. In contrast, XIAP-deficient cells from XLP2 patients do not show impaired NK cell mediated cytotoxicity [80, 172]. NK cell

degranulation after K562 stimulation is normal in most patients with XLP1 and XLP2 [186]. Flow cytometric analysis of intracellular SAP and XIAP, respectively, is a rapid test proven helpful in the diagnosis of XLP1 and XLP2, respectively [219, 220].

In summary, assessment of cytotoxic lymphocyte function and degranulation as well as flow cytometric analysis of intracellular perforin, SAP and XIAP are important tools in the diagnostics of primary HLH, and can guide the sequencing efforts.

4.4 CLINICAL FEATURES AND LABORATORY FINDINGS IN PRIMARY HLH

In this section clinical features and laboratory findings in the different groups of patients with primary HLH described in **Papers I, II, IV, and V** will be discussed. The findings are summarized in Table 3 (page 46).

4.4.1 Age at diagnosis of HLH

In **Paper I**, the median age at diagnosis of the 38 included patients with primary HLH was 5.1 months, ranging from 21 days to 14.7 years (mean = 28.5 months). In this study the underlying genetic defect was not studied, thus it was not possible to subgroup the patients genetically.

In **Paper II**, the clinical presentations of six GS2 patients in our cohort and, in addition, all patients with confirmed *RAB27A* mutation reported in the English literature up to March 1, 2009 (n=37, 18 patients omitted due to a paucity of clinical data), are described. The median age at diagnosis, among the 18 patients with available data, was 6.1 months, ranging from 1.8 months to 13.5 years (mean 31.5 months).

Among the eleven FHL5 patients described in **Paper IV**, the median age at diagnosis was 19 months, ranging from 3.5 months to 17 years (mean = 58 months), whereas the median age at onset was 15 months, ranging from 2 months to 17 years (mean = 53 months). Together with the FHL5 patients previously described by zur Stadt *et al.* [68], the median age at onset was 9 months ranging from 1.5 months to 17 years (mean = 30 months, n=23).

In **Paper V**, the median age at diagnosis among the eight FHL3 patients carrying the intron 1 mutation, c.118-308C>T, on at least one allele was 4.7 months, ranging from 1.8 months to 7.5 months (mean = 4.5 months). The only patient with this mutation in a homozygous state was diagnosed with HLH at the age of 4.4 months. Among the twelve patients with the 253 kb inversion straddling the *UNC13D* locus in a homozygous state, the median age at diagnosis was 3.4 months, ranging from 1 day to 15 months (mean = 5.2 months). The median age at diagnosis for FHL3 patients with bi-allelic disruptive *UNC13D* mutations has previously been reported to be 3 months [201]. Missense mutations in *UNC13D* have been associated with a later age at onset and milder defects in NK cell cytotoxicity and degranulation [176]. A partial restoration of NK cell cytotoxicity and degranulation, associated with a later onset of

HLH, are more commonly seen in FHL4 and FHL5 [69, 70, 161, 184]. However, in the patients carrying the inversion no difference was seen in the age at onset compared to other FHL3 patients, despite the partial restoration seen in NK cell cytotoxicity and degranulation.

4.4.2 Family history and consanguinity

In **Paper I**, 15 of the 38 patients (39 %) had familial disease, which was known at the time of diagnosis in ten of the patients. Three of the patients (8 %) were born to consanguineous parents. In **Paper II**, 21 of 35 GS2 patients (60 %) were reported with a familial disease, and 34 of 43 of the patients (79 %) were born to consanguineous parents. In **Paper IV**, six of ten FHL5 patients (60 %) had a familial disease and consanguinity was noted in three of ten patients (30 %). In **Paper V**, seven of 20 FHL3 patients (35 %) had a familial disease, whereas none of the 19 patients with available information were born to consanguineous parents.

4.4.3 Gender

In **Paper I**, there was a small male predominance in the patient cohort (21 of 38 patients, 55.3 %). A tendency of male predominance has previously been reported in other cohorts of primary HLH patients [111, 112]. This could hypothetically be addressed to an X-linked contribution in the underlying molecular defect. The underlying genetic defect was not studied, and XLP patients fulfilling the diagnostic criteria for HLH would be included in the study. This could result in a skewed gender ratio. However, also among the GS2 patients in **Paper II**, there was a male predominance, (24 of 43, 55.8 %). In contrast only 4 of 11 FHL5 patients (36 %) were of male origin in **Paper IV**. In **Paper V**, no skewed gender ratio was seen; ten of 21 patients (47.6 %) were of male origin. Of note, **Paper I** is the only population-based study among those included in this thesis.

4.4.4 Clinical presentation and laboratory findings in primary HLH

In **Paper I**, the most common presentations of HLH were fever, splenomegaly, bicytopenia, and elevated sCD25 reported in 35 of 35 (100 %), 36 of 38 (95 %), 38 of 38 (100 %), and eleven of eleven (100 %) of the patients with data available, respectively. Furthermore, 31 of 33 patients (94 %) had either elevated triglycerides and/or decreased fibrinogen, and 18 of 20 patients (90 %) had elevated ferritin. The only criterion that significantly changed in frequency over time was hemophagocytosis ($p=0.047$). Between 1987 and 1996, 20 of 21 patients (95 %) were reported with hemophagocytosis compared to eight of twelve patients (67 %) between 1997 and 2006. This can possibly be attributable to the previous view amongst many treating physicians that hemophagocytosis was almost obligatory for the diagnosis, whereas it is now well-known that hemophagocytosis is not always evident, especially not in the early phase of HLH [55]. In addition, the suspicion of HLH was earlier possibly raised more often by findings of hemophagocytosis while the diagnosis now more often may

be investigated for at an earlier phase of the disease. In addition to the manifestations included in the eight criteria, the majority of the patients shared elevated liver transaminases and bilirubin as well as hyponatremia.

Among the GS2 patients included in **Paper II**, fever, splenomegaly, and bicytopenia were also evident in the majority of patients, 36 of 38 (95 %), 30 of 35 (86 %), and 36 of 38 (95 %), respectively. sCD25 was reported elevated in the only patient tested. Seventeen of 21 patients (81 %) had elevated triglycerides and/or decreased fibrinogen, and 14 of 17 patients (82 %) had elevated ferritin. Hemophagocytosis was found in 16 of 24 patients (67 %).

In **Paper IV**, all ten patients who developed HLH were reported with fever and splenomegaly and nine of ten (90 %) had bicytopenia. Furthermore, all ten patients that developed HLH were reported with elevated triglycerides and/or decreased fibrinogen. The two patients evaluated for sCD25 both had pathologically elevated levels. Eight of nine patients (89 %) had elevated ferritin and six of ten patients (60 %) were reported with hemophagocytosis. In addition, nine of ten patients had elevated liver transaminases. In total, nine of the eleven FHL5 patients fulfilled the diagnostic criteria according to HLH-2004 [63]. One patient fulfilled 4 of 6 criteria evaluated and one patient was a healthy sibling with a confirmed genetic diagnosis.

In **Paper V**, nineteen of the patients (90 %) were reported with fever, 20 of 21 (95 %) had splenomegaly, and 18 of 21 (86 %) had bicytopenia. Elevated triglycerides and/or decreased fibrinogen were seen in 19 of 21 (90 %) patients, 18 of 21 patients (86 %) had elevated ferritin and hemophagocytosis was noted in 10 of 20 patients (50 %). sCD25 was elevated in all ten patients evaluated. In all, 19 of 21 patients fulfilled the diagnostic criteria according to HLH-2004 [63], the remaining two patients both had a familial disease.

4.4.5 Neurological manifestations

In **Paper I**, 20 of 36 patients (56 %) of the patient displayed neurological alterations during the course of disease, whereas pathologic CSF was noted in 19 of 25 patients (76 %). Together, 26 of 36 patients (72 %) were reported with neurological alterations and/or pathologic CSF. The most commonly reported neurological alteration was seizures.

In **Paper II**, 21 of 38 (55 %) were reported with neurological alterations at diagnosis, and 29 of 43 patients (67 %) were reported with neurological alterations during the course of disease. Among the neurological alterations described, seizures and cranial nerve palsy were the most commonly reported. Pathologic CSF was noted in six of eleven patients (55 %). Data regarding CSF analyses were missing in the remaining 32 patients. Interestingly, patients with reported onset/diagnosis at 12 months of age or above had a significantly higher frequency of neurological alterations compared to those with reported onset/diagnosis below 12 months of age ($n=38$, $p=0.005$). Furthermore, trends towards a lower proportion of the patients with a previously

affected relative were reported with neurological alterations (36 %) compared to those without (72 %, n=29, p=0.12).

In **Paper IV**, neurological symptoms were observed in five of ten of the FHL5 patients (50 %) described. In two of these patients, irritability was the only neurological symptom. In addition one patient had a hearing deficiency of unknown origin, and one patient had an elevated CSF cell count but displayed no neurological symptoms.

In **Paper V**, neurologic alterations were noted in eleven of 20 patients (55 %), and 15 of 18 patients (83 %) had pathological CSF with pleocytosis and/or elevated protein levels during the course of disease.

Taken together, many of the patients included in this thesis presented with neurological manifestations consistent with previous reports and knowledge of HLH. Of note, neurological manifestation may be the initial presentation of HLH and may dominate the course of disease [57, 221]. A high proportion of the GS2 patients in **Paper II**, 55 %, displayed neurological alteration at diagnosis, compared to 37 % of the patients with HLH reported by Horne *et al.* [58]. However, the facts that one third of the GS2 patients were without neurological alterations and that a previously affected relative may reduce the frequency of neurological alterations suggest that the neurological alterations are secondary to the inflammatory HLH state rather than a result of Rab27a-deficiency in the CNS. Similarly, Pachlopnik Schmid *et al.* argued that the neurological symptoms in GS2 are secondary to the HLH in a single-center study describing 10 patients with GS2 [222]. In contrast to this, the CNS disease in patients with CHS may progress after a successful HSCT, most likely due to a continuous lysosomal defect in neurons and glial cells [223].

A review of CNS involvement in primary HLH was recently published by Deiva *et al.*, describing neurological manifestations at disease onset in 29 of 46 (63 %) patients with primary HLH who had undergone a neurologic evaluation and magnetic resonance imaging (MRI) within 2 weeks and 6 months of HLH diagnosis, respectively [224]. Twenty-three of the patients (50 %) had abnormal CSF at HLH onset [224]. The frequency of patients with genetically determined FHL with neurological symptoms at diagnosis was similar to what was seen in patients with other types of primary HLH [224]. As described above, Horne *et al.* have previously reported 72 of 194 (37 %) patients with neurological symptoms at diagnosis of HLH. In comparison with the study by Deiva *et al.*, the cohort reported by Horne *et al.* had a higher mean age at onset and the patients were recruited from the multi-center study HLH-94. These facts may partly explain the discrepancy in the proportion of patients with neurological symptoms [58].

In summary, neurological manifestations are common among patients with HLH. Many patients display neurological manifestations at onset of HLH, while other develops CNS disease during the course of disease. Thus, a correct diagnosis of HLH and prompt initiation of treatment is important in order to reduce the CNS disease and late effects often seen in patients with primary HLH.

4.4.6 Less common findings in primary HLH

Among the six patients with GS2 in our cohort presented in **Paper II**, three were diagnosed with GS2 within our study. All three siblings were fair-haired but not considered abnormal by the treating physicians. Corroborating the genetic diagnosis, examination of hair shafts by light microscopy and electron microscopy revealed abnormal accumulation of pigment compared to what was seen in a healthy fair-haired child. Furthermore, two of the three additional patients were first diagnosed as having FHL and first later diagnosed with GS2. The partial albinism with silvery hair can easily be over-looked, especially in the Nordic population where fair hair is common. Hence, it is important to consider GS2 among patients with HLH.

Interestingly, the data in **Paper IV** suggest that FHL5 can be associated with symptoms not typically seen in FHL patients. Gastrointestinal symptoms, hypogammaglobulinemia, and bleeding disorders were observed in approximately one third of the patients. The index patient in the study presented with an inflammatory bowel-like disease. Fecal calprotectin was elevated and colonoscopy revealed diffuse inflammation from cecum to the sigmoid colon, suggesting a possible diagnosis of inflammatory bowel disease. The colitis improved after treatment with oral mesalazin. This prompted us to review gastrointestinal symptoms in the other patients in the study. Two siblings both had chronic diarrhea, and other patients were observed with minor symptoms such as gastroesophageal reflux, vomiting, inflammation of lips and oral mucosa, and recurrent abdominal pain. Corroborating our findings, Pagel *et al.* in 2012 reported that severe chronic diarrhea was observed in 14 of 37 FHL5 patients (38 %) [195]. The diarrhea was often present before the onset of HLH and persisted in at least six of eight patients who had undergone a HSCT [195]. Of interest, they observed a clear association between the gastrointestinal symptoms and patients not carrying the common splice site mutation, 1247-1G>C, suggesting that patients with that mutation have a remaining Munc18-2 function. The gastrointestinal symptoms observed in FHL5 patients could be attributed to the impaired immunity and general hyperinflammatory state in HLH. Many other immunodeficiencies, including Wiscott-Aldrich syndrome and SCID, are also associated with gastrointestinal symptoms [82, 225]. Likewise, XLP2 patients commonly present with colitis [52]. An alternative explanation is that gastrointestinal symptoms are secondary to a loss of function of the Munc18-2 protein in cell types other than cytotoxic lymphocytes. The initial reports of Munc18-2 described a widespread expression in epithelial tissues, such as the intestines [226-228], and Munc18-2 could thus hypothetically act to maintain epithelial integrity.

One patient in **Paper IV** displayed ecchymosis and a tendency of severe, prolonged bleeding. Furthermore, two siblings both had a prolonged coagulation profile, thrombocytopenia, and displayed petechiae. The notion of a possible bleeding disorder among FHL5 patients was also made by Pagel *et al.*, reporting eight of 37 patients to have bleeding problems [195]. Several studies have reported a role of different Munc18 isoforms in platelet function [229, 230]. Four FHL5 patients were identified with a secretion defect in platelets [231]. However, only one of those evolved a severe bleeding disorder [231]. Further studies might improve the understanding of a possible complicating bleeding disorder among FHL5 patients.

Hypogammaglobulinemia was noted among three of eleven patients (27 %) of the FHL5 patients in **Paper IV**, and among nine of 37 (24 %) in the report by Pagel *et al.* [195]. The hypogammaglobulinemia was typically seen after EBV-induced HLH episodes. Of note, hypogammaglobulinemia was also noted among FHL3 patients in the study by Pagel *et al.* [195]. Typically, hypogammaglobulinemia is seen in XLP1 and XLP2, but seldom reported in FHL patients [80, 232, 233]. Some FHL5 patients have been demonstrated to have a loss in B-cell maturation, and a role of Munc18-2 in the B cell to T cell communication has been suggested [195]. However, it is still unclear how the loss of Munc18-2 contributes to the demise of humoral immunity in FHL5.

One of the patients in **Paper IV** had a hearing deficiency of unknown origin. Interestingly, sensorineural hearing loss later was described in several FHL5 patients [195]. These patients was affected by a low-frequency hearing impairment, that easily can remain undetected [195]. It is unclear whether the deficiency is secondary to the inflammatory process or associated with a defective signal transduction mechanism of the inner ear [195]. Sensorineural hearing loss has previously been described in a perforin-deficient patient [234], but is usually not observed in FHL patients.

4.4.7 Outcome

In **Paper I**, altogether 14 of 38 patients (37 %) were alive at the time of evaluation. The great majority (34 of 38, 89 %) had been treated with a regimen including epipodophyllotoxin derivatives (etoposide, teniposide) during the course of disease. Between 1997 and 2006, all patients but one were treated according to the HLH-94 or HLH-2004 protocols [62, 63]. All patients alive have undergone a HSCT.

Divided into two ten-years periods, a higher proportion of the patients survived until a HSCT in the second period, nine of 22 patients (41 %) 1987-1996 compared to 13 of 16 patients (81 %) 1997-2006 ($p=0.013$, Pearson Chi-square test). Furthermore, an improved outcome after HSCT was seen the second period, even though not statistically significant, with 56 % mortality (five of nine patients) 1987-1996 compared to 23 % mortality (three of 13 patients) 1997-2006 ($p=0.187$, Fisher's Exact Test 2-sided). The overall survival was significantly higher the second period 1997 to 2006 (ten of 16 patients, 63 %) compared to 1987 to 1996 (four of 22 patients, 18 %, $p=0.005$, Pearson Chi-square test). Among the patients diagnosed 1997-2006, the time period most comparable to HLH-94, the overall survival was slightly higher compared to the 5-year probability-of-survival reported in HLH-94 ($54\pm6\%$) [111]. The final report of HLH-2004 is not yet published. The improved survival the second 10-year period in **Paper I** could be attributable an increased awareness of primary HLH in Sweden, with improved diagnostics and treatment, including HSCT.

Among all the GS2 patients reviewed in **Paper II**, 19 of 42 were alive (45 %). Of the six patients with GS2 in our cohort of patients all were alive at the time of evaluation. In **Paper IV**, nine of the eleven patients (82 %) described were alive. Two of the patients died before HSCT, whereas the third patient died of a reactivated adenovirus infection after a haploidentical HSCT. Of the eight patients alive, five have been

successfully transplanted. At the time of publication three patients were alive without HSCT. The possible prospective risk of hematological malignancy, high susceptibility of infections, and reactivation of HLH and neurological disease were discussed in the paper. Of importance, one of these non-transplanted patients developed an EBV-positive classical Hodgkin lymphoma (cHL) 45 months after the clinical presentation of HLH (Machaczka *et al.* submitted). Of note, this patient did not present with HLH at the time of lymphoma development. The cumulative dose of etoposide given during HLH-therapy was low (0.2 g/m²). Similarly, Pagel *et al.* reported a patient, homozygous for the common splice site mutation in *STXBP2*, c.1247-1G>C, that developed cHL and HLH features at the age of 9 years [195]. These findings are of great importance in a clinical setting, suggesting that hypomorphic *STXBP2* mutations might result in lymphoma development. This risk must be taken into account in the decision regarding HSCT in patients with defined disease-causing mutations in FHL genes. In **Paper V**, 16 of 21 FHL3 patients were treated according to the HLH-2004 protocol, four were treated according to the HLH-94 protocol and one patient went directly to HSCT. Thirteen of 21 patients (62 %) are alive after a HSCT.

It is difficult to compare the outcome in the different cohorts of patients with primary HLH reported in this thesis. The number of patients included in each cohort is limited and patients are treated at different centers in different time periods. The advances in the genetic and functional diagnostics of primary HLH will enable sub-categorization of patients with HLH and could possibly, together with other factors, be used in risk stratification for more individualized treatment in the future.

Table 3. Clinical data in the patients studied related to the period of diagnosis.

	Paper I (%)	Paper II (%)	Paper IV (%)	Paper V (%)
Diagnosis	Primary HLH	GS2	FHL5	FHL3
Total number of patients	38	43 (6+37*)	11	21
Age at diagnosis				
<3 months	13 of 38 (34)	3 of 18 (17)	1 of 10 (10)	8 of 21 (38)
3-11.9 months	10 of 38 (26)	8 of 18 (44)	4 of 10 (40)	10 of 21 (48)
1-2.9 years	5 of 38 (13)	1 of 18 (6)	1 of 10 (10)	3 of 21 (14)
3-14.9 years	10 of 38 (26)	6 of 18 (33)	4 of 10 (40)	
Median age at diagnosis	5.1 m	6.1 m	19 m	4.7 m [#] /3.4 m [§]
Range age at diagnosis	21 d-14.7 y	1.8 m-13.5 y	3.5 m-17 y	1.8 m-7.5 m [#] / 1 d-15 m [§]
Familial disease	15 of 38 (39)	21 of 35 (60)	6 of 10 (60)	7 of 20 (35)
Consanguinity	3 of 38 (8)	34 of 43 (79)	3 of 10 (30)	0 of 19 (0)
Male gender	21 of 38 (55)	24 of 43 (56)	4 of 11 (36)	10 of 21 (48)
Fever	35 of 35 (100)	36 of 38 (95)	10 of 10 (100)	19 of 21 (90)
Splenomegaly	36 of 38 (95)	30 of 35 (86)	10 of 10 (100)	20 of 21 (95)
Bicytopenia	38 of 38 (100)	36 of 38 (95)	9 of 10 (90)	18 of 21 (86)
Triglycerides ↑ / Fibrinogen ↓	31 of 33 (94)	17 of 21 (81)	10 of 10 (100)	19 of 21 (90)
Ferritin ↑	18 of 20 (90)	14 of 17 (82)	8 of 9 (89)	18 of 21 (90)
Soluble CD25 ↑	11 of 11 (100)	1 of 1 (100)	2 of 2 (100)	10 of 10 (100)
Hemophagocytosis	28 of 33 (67)	16 of 24 (67)	6 of 10 (60)	10 of 20 (50)
Neurological alterations during the course of disease	20 of 36 (56)	29 of 43 (67)	5 of 10 (50)	11 of 20 (55)
Pathological CSF**	19 of 25 (76)	6 of 11 (55)		15 of 18 (83)
Outcome - proportion alive	14 of 38 (37)	19 of 42 (45)	9 of 11 (82)	16 of 21 (62)

* = patients with *RAB27A* mutations described in the English literature up to 1 March 2009, d=days, m=months y=years, [#] = patients with ≥ 1 allele with the intron 1 mutation (c.118-308C>T), [§]Homozygous 253-kb inversion straddling the *UNC13D* locus, ** CSF = cerebrospinal fluid,

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The papers included in thesis aimed to increase the clinical, genetic and biological understanding of HLH. The main conclusions are summarized below.

- The annual incidence of primary HLH in Sweden 1987-2006 was estimated to be 1.2 per million children aged less than 15 years, corresponding to 1.8 per 100 000 live born children.
- The annual incidence was higher in the northern part of Sweden (“Norrland”) compared to the middle and southern part of Sweden (“Svealand” and “Götaland”), 2.1 compared to 1.0 per million children below the age of 15 years.
- A minimal annual incidence based on defective cytotoxic lymphocyte function and genetic defects indicative of primary HLH the subsequent 5-year period, 2007-2011, was 1.5 per million children below the age of 15 years, corresponding to 2.2 per 100 000 live born.
- One of 21 families diagnosed as having FHL was identified with bi-allelic mutations in the *RAB27A* gene consistent with the diagnosis of GS2. This highlights the importance of considering GS2 among patients with HLH, especially in fair-haired individuals where the partial albinism easily can be overlooked.
- Neurological alterations were reported in a high proportion of patients with GS2, 55 % at diagnosis and 67 % during the course of disease. In comparison Horne *et al.* reported 37 % of patients with HLH to display neurological alterations at diagnosis.
- Rab27a is required for degranulation induced by receptors both for natural cytotoxicity and ADCC. However, defects in cytotoxicity and degranulation were less profound in GS2 patients compared to FHL3 patients, consistent with late onset and long periods of remission sometimes seen in GS2 patients.
- Activation of NK cells with PMA and ionomycin result in induced myosin-dependent colocalization of Munc13-4 and Rab27a with perforin. A similar increase in colocalization occurs after incubation with K562 cells.
- There is an inverse relationship between Rab27a and Munc13-4 where recruitment of Rab27a to perforin-containing granules is Munc13-4 dependent and vice versa.
- Engagement of LFA-1, NKG2D, and 2B4 by their ligand ICAM-1, ULBP1, and CD48, respectively, induce colocalization of Rab27a but not Munc13-4

with perforin, whereas engagement of CD16 by IgG induces colocalization of Munc13-4 but not Rab27a with perforin. Thus, engagement of different receptors regulates distinct events for lytic granule maturation.

- A highly variable disease severity was observed in our cohort of FHL5 patients, with an age at onset ranging from 2 months to 17 years. Furthermore, about a third of the patients presented with gastrointestinal symptoms, bleeding disorder, or hypogammaglobulinemia. Thus, the presentation of FHL5 can be diverse and the diagnosis should also be considered in patients with manifestations other than those typically associated with HLH.
- NK cell cytotoxicity and degranulation were defective in all FHL5 patients evaluated. Notably, IL-2 stimulation partially restored the defect, as previously seen in FHL4 patients.
- The identification of mutations in HLH-associated genes in late-onset patients with an atypical presentation raises questions regarding treatment. A possibly increased risk of hematological malignancies over time, and the risk that an HLH relapse sometimes may be complicated with neurological alterations, must be taken under consideration if HSCT is postponed.
- The point mutation, c.118-308C>T, located in the intron 1 of *UNC13D*, selectively impairs *UNC13D* transcription in lymphocytes and was identified in patients spread over Europe.
- A 253 kb inversion straddling the *UNC13D* locus was identified in patients of Scandinavian origin. The mutation affects the 3' end of the transcript and abolishes Munc13-4 expression.
- The identification of two non-coding aberrations causative of FHL3 provides a molecular diagnosis for many patients of northern European origin and highlights aberrations outside the coding regions as a cause of disease. Our findings make a case for sequencing of evolutionary conserved regions in the diagnostics of primary immunodeficiency syndromes.

Together, the findings in this thesis have provided a molecular diagnosis for the great majority of children with a typical form of primary HLH in Sweden, as well as many patients in other parts of Scandinavia and other countries. Identification of the underlying genetic defect is of great value in the diagnostics of HLH, which often can be challenging. An early diagnosis is important for prompt initiation of adequate treatment and thus also for an improved outcome and reduced late effects, including neurological alterations. Moreover, Guthrie-card based neonatal screening for the common 253 kb inversion is currently being discussed in Sweden in order to identify patients with FHL3 before onset of HLH. In addition, a genetic diagnosis provides the possibility of carrier testing, prenatal diagnosis, and preimplantation genetic diagnosis for the affected families.

The findings in this thesis also include epidemiological data about the incidence of primary HLH in Sweden, and describe the clinical presentation and laboratory findings, including NK cell function, in different subcategories of patients with HLH.

Lymphocyte cytotoxic function can, simplified, be viewed as a continuous range, where patients with truncating mutations in HLH-associated genes constitute one end of the spectrum, with a complete absent function. Hypomorphic mutations, such as missense mutations and splice site mutations in genes associated with primary HLH, are examples where a reduced, but not fully absent, lymphocyte cytotoxic function is observed. These mutations have been associated with later age at onset and a more atypical presentation of disease. Based on the fact that the clinical presentation of primary HLH can vary considerably, and include symptoms not typically associated with HLH, an increased awareness of HLH is needed not only among clinicians within the field of pediatric hematology but also for adult hematologists, infectious disease specialists, gastroenterologists, and intensivists. Furthermore, the knowledge of the diverse presentation of primary HLH stresses the difficulties in the discrimination between primary and secondary HLH, which is of importance in regards to treatment and the decision of HSCT.

To further increase the understanding of HLH, and other immunodeficiencies, continued translational research is needed, with a close collaboration between clinicians and researchers. With more fine-tuned assessment of functional defects in the immune system, together with the sequencing advances in the field of genetics, many novel primary immunodeficiencies will likely be revealed in the near future. Whole exome sequencing is now frequently used and whole genome sequencing will add on further with the possibility to detect non-coding variants, shown in this thesis to be important at least in the case of primary HLH. The great challenge will be to interpret all data in this complex field of human immunity. Hopefully, improved diagnostics and sub-categorizing of patients can catalyse a more individualized treatment in HLH and other immunodeficiencies.

The work with this thesis has been very rewarding and has for me been a great example of translational research and collaboration between different fields of science. It has also provided close contact with many treating clinicians and sometimes also with the affected families. My sincere hope is to be involved both in the research and in a clinical setting of this interesting field in the future.

6 SVENSK SAMMANFATTNING

Denna avhandling omfattar studier av hemofagocyterande lymfohistiocytos (HLH). Målsättningen har varit att öka den kliniska, genetiska och biologiska kunskapen av HLH för att på så sätt förbättra diagnostik och överlevnad för drabbade individer.

HLH innebär ett livshotande, hyperinflammatoriskt tillstånd med symptom såsom feber, låga blodvärden samt lever- och mjältförstoring. HLH kan även drabba det centrala nervsystemet och ge svåra neurologiska symptom med kramper och förlamning. HLH delas vanligtvis in i två olika grupper, medfödd/primär HLH och förvärvad/sekundär HLH. I gruppen primär HLH ingår familjär hemofagocyterande lymfohistiocytos (FHL) som nedärvs autosomalt recessivt. Detta innebär att föräldrar som är bärare av ett sjukdomsorsakande anlag har 25 % risk att få ett sjukt barn. FHL är dödlig utan adekvat terapi. Behandlingen innefattar vanligen två steg. Först dämpas den intensiva hyperinflammationen med cellgifter och kortison. I ett andra steg görs en hematopoietisk stamcellstransplantation, vilket är nödvändigt för bot av den ärftliga formen av HLH. Med nuvarande behandling överlever drygt 50 % av individer drabbade av HLH. Hittills finns fyra gener kopplade till FHL (*PRF1*, *UNC13D*, *STX11*, och *STXBP2*). Defekter i dessa gener leder till avsaknad av, eller nedsatt, cytotoxicitet (cellers förmåga att döda målceller). Cytotoxiciteten är viktig i försvaret mot virusinfektioner och tumörceller samt även i nedregleringen av det egna immunförsvaret. Sjukdomsbilden vid HLH kan variera och det är därför ibland svårt att komma fram till rätt diagnos. Vidare kan det klinisk vara omöjligt att särskilja primär HLH och sekundär HLH. Ökad genetisk och biologisk förståelse av HLH är därför av största vikt för en korrekt diagnos och för planering av behandling.

År 1991 beskrev vår grupp att primär HLH drabbade 1.2 av 1 000 000 barn under 15 år. Sedan dess har kunskapen om FHL ökat och vi hypotiserade därför att den sanna incidensen kunde vara högre än tidigare estimerat. I den första delen av **delarbete I** visar vi dock, något överraskande, att den årliga incidensen av sjukdomen är oförändrad, 1.2 per 1 000 000 barn under 15 år vilket motsvarar 1.8 barn per 100 000 levande födda. I Sverige innebär det att ungefär 2 barn per år drabbas av primär HLH. I den andra delen av **delarbete I** beskriver vi genetiska analyser och studier av Natural Killer (NK) cellers cytotoxicitet som diagnostiska hjälpmedel för att hitta patienter med primär HLH.

Många individer med primär HLH har varit utan en genetisk diagnos, detta gäller särskilt individer av nordiskt ursprung. En av de viktigaste målsättningarna med denna avhandling var därför att hitta den underliggande genetiska orsaken till HLH hos dessa individer. Patienter med Griscelli syndrom typ 2 (GS2) drabbas också av HLH men uppvisar i tillägg till immunbristen en partiell albinism. I **delarbete II** visar vi att en av 21 familjer som fått diagnosen FHL istället var drabbade av GS2 med mutationer i genen *RAB27A*. Ytterligare tre patienter var initialt diagnostiserade med FHL och fick först senare den korrekta diagnosen GS2. Detta visar att det är viktigt att komma ihåg GS2 hos patienter med primär HLH eftersom den partiella albinismen kan vara svår att upptäcka. I **delarbete II** presenterar vi även en sammanställning av alla GS2 patienter med mutationer i *RAB27A* som har rapporterats i den medicinska litteraturen.

Delarbete III innehåller studier av funktionen av Rab27a (proteinet som kodas av genen *RAB27A*) och Munc13-4 (proteinet som kodas av genen *UNC13D*) i NK celler. Båda dessa

proteiner behövs för en normal NK-cellsfunktion. Vi visar även hur aktivering av specifika receptorer på NK-celler yta påverkar vad som händer med dessa proteiner i cellen och hur Rab27a och Munc13-4 är beroende av varandra för en korrekt funktion.

I **delarbete IV** beskriver vi sjukdomsbild, mutationsspektrum och NK-cellsfunktion hos patienter med FHL som orsakas av mutationer i genen *STXBP2*. Sjukdomsbilden är väldigt varierade och debutålder sträcker sig från 2 månader till 17 år. Många av patienterna uppvisade symptom som inte vanligtvis ses vid HLH, såsom besvär från mag-tarmkanalen, blödningsbenägenhet och låga nivåer av antikroppar i blodet. Det är därför viktigt att komma ihåg FHL som diagnos även hos patienter med en annorlunda, atypisk, sjukdomsbild.

I **delarbete V** beskriver vi två olika förändringar utanför de kodande delarna av genen *UNC13D* som en vanlig orsak till FHL i Sverige och övriga Europa. Vid konventionell genetisk analys undersöks enbart de kodande delarna och dessa förändringar har därför tidigare inte upptäckts. Dessa två förändringar, en punktmutation i intron 1 och en inversion med ena brottspunkten i intron 30, gör att inget Munc13-4-protein bildas och orsakar således en defekt NK-cellsfunktion.

Sammanfattningsvis så har fynden i denna avhandling gjort att den absoluta majoriteten av barn med en typisk form av primär HLH i Sverige, samt även många patienter utanför Sverige, kan få en genetisk diagnos som förklarar sjukdomen. Vidare har resultaten i denna avhandling gett ny kunskap om hur vanligt primär HLH är i Sverige samt om sjukdomspanorama och NK-cellsfunktion i olika grupper av patienter med primär HLH. En genetisk diagnos, tillsammans med analys av NK-cellers funktion, underlättar handläggningen av patienter med HLH och är således av största vikt för insättande av livräddande behandling. Tidig diagnos och behandling kan också minska risken för kvarstående neurologiska symptom hos drabbade individer. En genetisk diagnos möjliggör också fosterdiagnostik och anlagstestning av syskon och släktingar vilket kan vara till stor nytta för drabbade familjer.

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